Ministry of Economic Development
Manatū Ōhanga
Intellectual Property Office of New Zealand

PCT/NZ2004/000197

CERTIFICATE

This certificate is issued in support of an application for Patent registration in a country outside New Zealand pursuant to the Patents Act 1953 and the Regulations thereunder.

I hereby certify that annexed is a true copy of the Provisional Specification as filed on 23 October 2003 with an application for Letters Patent number 529153 made by ANTIPODEAN BIOTECHNOLOGY LIMITED.

Dated 1 September 2004.

Neville Harris
Commissioner of Patents, Trade Marks and Designs

COMMISSIONER OF PATENTS, TRADEMARKS & DESIGNS
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529153

NEW ZEALAND

PATENTS ACT, 1953

PROVISIONAL SPECIFICATION

MITOCHONDRIALLY TARGETED ANTIOXIDANTS

We, ANTIPODEAN BIOTECHNOLOGY LIMITED, Level 2, 16 Viaduct Harbour Avenue, Auckland, do hereby declare this invention to be described in the following statement:

The invention relates to antioxidants having a lipophilic cationic group, their synthesis and physicochemical properties that favour their use as, for example, pharmaceuticals.

Oxidative stress contributes to a number of human degenerative diseases associated with ageing, such as Parkinson's disease, and Alzheimer's disease, as well as to Huntington's Chorea and Friedreich's Ataxia, and to non-specific damage that accumulates with aging. It also contributes to inflammation and ischaemic-reperfusion tissue injury in stroke and heart attack, and also during organ transplantation and surgery. To prevent the damage caused by oxidative stress a number of antioxidant therapies have been developed. However, most of these are not targeted within cells and are therefore less than optimally effective. Moreover, many such antioxidants have unfavourable physicochemical properties that limit their bioavailability, and their ability to penetrate to the target organ to exert a therapeutic effect.

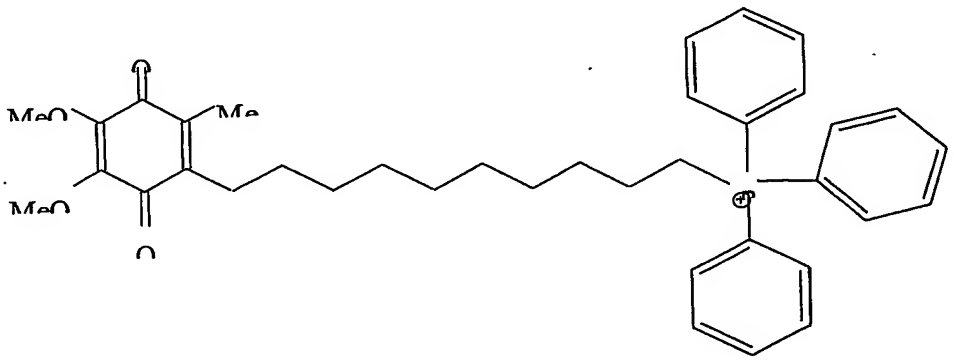
Mitochondria are intracellular organelle responsible for energy metabolism. Consequently, mitochondrial defects are damaging, particularly to neural and muscle tissues which have high energy demands. They are also the major source of the free radicals and reactive oxygen species that cause oxidative stress inside most cells. Therefore, the applicants believe delivering antioxidants selectively to mitochondria will be more effective than using non-targeted antioxidants. Accordingly, it is towards the provision of antioxidants which may be targeted to mitochondria that the present invention is directed.

Lipophilic cations may be accumulated in the mitochondrial matrix because of their positive charge (Rottenberg, 1979 *Methods Enzymol* **55**, 547. Chen, 1988 *Ann Rev Cell Biol* **4**, 155). Such ions are accumulated provided they are sufficiently lipophilic to screen the positive charge or delocalise it over a large surface area, also provided that there is no active efflux pathway and the cation is not metabolised or immediately toxic to a cell.

The focus of the invention is therefore on an approach by which it is possible to use the ability of mitochondria to concentrate specific lipophilic cations to take up linked antioxidants so as to target the antioxidant to the major source of free radicals and reactive oxygen species causing the oxidative stress.

Examples of antioxidant compounds that show good antioxidant activity yet exhibit poor bioavailability with respect to the target compartment *in vivo* include Coenzyme Q (CoQ) and Idebenone. Both of these compounds must be administered at very high dose rates to be efficacious, and therefore have low therapeutic efficacy when referenced to the dose rate administered.

In US Patent No. 6331532 by reference to exemplifications of compounds mitoquinol and mitoquinone (referred to collectively herein as mitoquinone/mitoquinol) there is disclosed the prospect of mitochondrial targeting of an antioxidant moiety reliant upon a lipophilic cation covalently coupled to the antioxidant moiety. The exemplified compound (1) therein (despite generalisation of the bridge length), is the compound mitoquinone of the formula



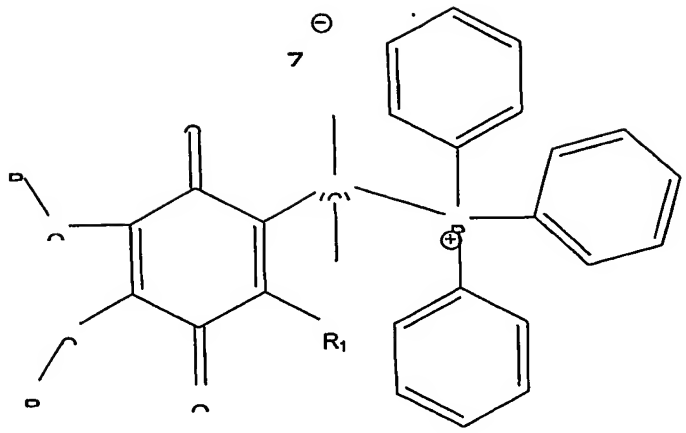
(1)

with a carbon bridge length of 10 (i.e., C_{10} bridged). Its reduced form, mitoquinol, is also C_{10} bridged.

Mitoquinone/mitoquinol, despite excellence in antioxidant activity and targeting and accumulation in mitochondria *in vitro* and *in vivo*, we have found to be somewhat unstable as the bromide salt.

We believe without wishing to be bound by any theory that for an antioxidant compound, activity *in vitro* (whether antioxidant activity, or mitochondrial accumulation) is by no means the sole determinant of efficacy *in vivo* and/or of therapeutic efficacy. Whilst it is true that, to be useful as a mitochondrially-targeted antioxidant compound of the present invention, an antioxidant compound must exhibit a suitably high antioxidant activity *in vitro*, to be efficacious *in vivo* the mitochondrially targeted antioxidant compounds must exhibit other desirable physicochemical properties, for example, suitably high bioavailability and/or suitable stability.

We have determined that mitoquinone (1)/mitoquinol has a moderately high partition coefficient (eg., about 160 when assessed by an octanol:water partition system, see herein), that idebenone has a high partition coefficient of 3.1×10^3, and ubiquinone (CoQ_{10}) has a very high partition coefficient of 1.8×10^{20}.



We believe compounds of the general formula I

I

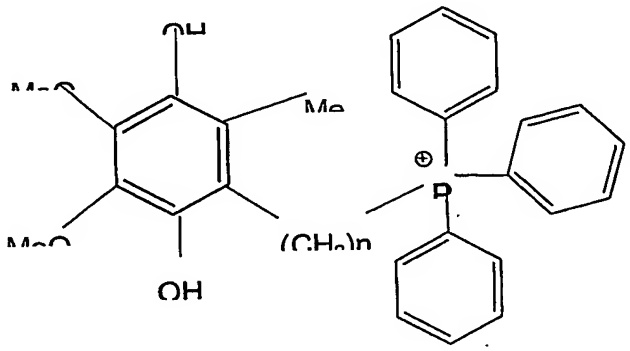
where the bridge length is less than about C_{15} (and preferably less than C_7, more preferably less than C_5) will have low partition coefficients which are in the same order as those of derivatives of CoQ such as CoQ_0, yet surprisingly may provide *in vivo* when orally administered mitochondrial targeting of antioxidant activity many times beyond what would be expected from the *in vitro* studies of mitochondrial targeting of antioxidant activity, and many times beyond that observed with, for example, derivatives of CoQ such as CoQ_0. By way of example, when the bridge of the mitochondrially targeted antioxidant compound is C_5, the partition coefficient of the crystalline compound (Mitoquinone-C5) is 2.8 ± 0.3, *i.e.*, much less than that of the C_{10} compound.

We believe without wishing to be bound by any theory that at least in part by virtue of their low partition coefficient the mitochondrially-targeted antioxidant compounds of the present invention exhibit advantageous bioavailability and/or mitochondrial targeting and accumulation *in vivo* and are thereby therapeutically efficacious at low dose rates in comparison to other antioxidant compounds.

A lower partition coefficient is in our view desirable for greater bioavailability particularly where administration is to be oral and/or where there is targeting of the antioxidant compound to mitochondria in the tissues of internal organs (eg., brain, heart or other organs). Conversely, we believe compounds which exhibit high partition coefficients are

inappropriate for delivery orally for the treatment of oxidative stress where there is a requirement for oral bioavailability, organ penetration, and for passage through a barrier such as that of the blood brain barrier.

In PCT/NZ02/00154 there is disclosed a process for manufacturing compounds of the general



formula II

II

Such a procedure we have found is inappropriate for chain lengths or for bridging groups less than C_6 (ie., where $n<6$).

The present invention recognises therefore an advantage in being able to prepare compounds of bridge length C_6 or less.

We have found on preparation of compounds of bridge length less than C_6 that such compounds have distinct and desirable physicochemical properties from those taught within US 633152 and PCT/NZ02/00154.

Advantageously, examples of compounds of the present invention are crystalline and/or solid in form, which amongst other advantages renders them particularly suitable to formulation (eg., by tableting or encapsuling) in orally-administerable dosage forms and pharmaceutical formulations. This, in conjunction with a low partition coefficient (preferably less than about 20, more preferably less than about 10, still more preferably less than about 5), results in novel mitochondrially-targeted antioxidant compounds with desirable physicochemical properties particularly suited to therapeutic pharmaceutical use.

We have also determined a desirability to offer examples of the mitochondrially targeted antioxidant compounds of the present invention with their positive charge in

association with a suitable anion thereby to provide the compound as a general neutralised solid or crystalline product. In such salt forms however certain salt forming anions we have found to be best avoided as they exhibit reactivity against the antioxidant moiety, and/or may lead to cleavage at or of the antioxidant moiety, or are considered pharmaceutically undesirable. For example, nitrate moieties are considered inappropriate generally by pharmaceutical companies as being pharmaceutically or environmentally unacceptable whilst a hydrogen bromide frequently used in salt forming of such compounds we find to have nucleophilic properties that can lead to a reactivity against the antioxidant moiety, for example, a cleavage of a methyl group from the antioxidant moiety of the compound of general formula (2) herein, and/or some overall decrease in stability of the overall compound. For example, we have determined that the bromide salt of compound 1 is somewhat unstable

We believe therefore that salt forms, including salt forms as a solid or crystalline form, of mitochondrially targeted antioxidants are best associated with an anion or like moiety that is not nucleophilic, or one which does not exhibit reactivity against the antioxidant moiety. It is also preferable that the anion is pharmaceutically acceptable.

In a first aspect, the present invention consists in a compound comprising a cationic species being a lipophilic cationic moiety covalently coupled to an antioxidant moiety, and an anionic complement for said cationic moiety, wherein

i) the cationic species is capable of mitochondrially targeting the antioxidant species, and

ii) the anionic complement is not HBr, and/or

iii) the anionic complement is non-nucleophilic, and/or

iv) the anionic complement does not exhibit reactivity against the antioxidant moiety.

Conveniently, the anion is selected from the group consisting of alkyl or aryl sulfonates or nitrates.

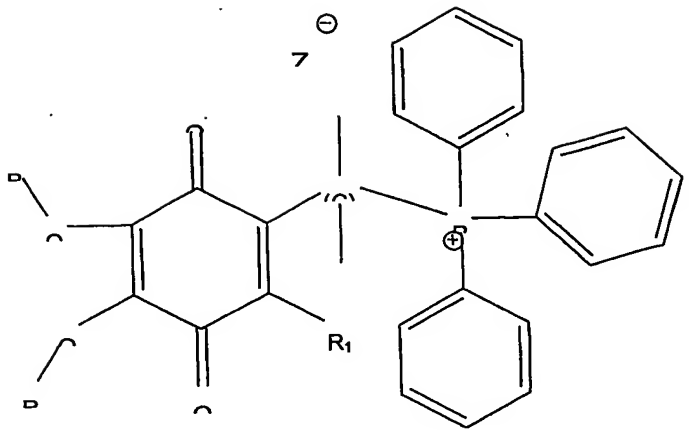
Preferably the anion is methanesulfonate.

The lipophilic cation may be a substituted triphenylphosphonium cation.

Alternatively, the lipophilic cation may be an unsubtituted triphenylphosphonium cation.

Preferably the antioxidant moiety is a quinone or a quinol.

In one embodiment the compound has the general formula III



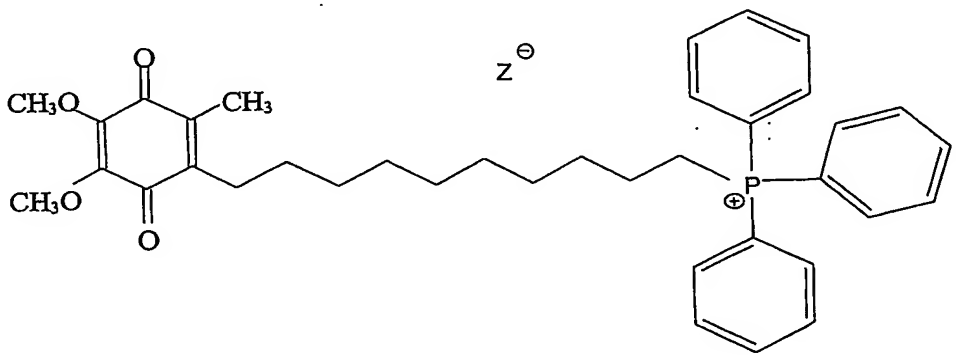
III

and/or its quinol form, wherein R_1, R_2, and R_3, which can be the same or different, are selected from C_1 to C_5 alkyl (optionally substituted) moieties or H, and wherein n is an integer from 2 to 10, and wherein each C of the (C)n bridge is saturated, and wherein Z is a non nucleophilic anion.

Preferably Z is selected from the group consisting of alkyl or aryl sulfonates or nitrates.

More preferably Z is methanesulfonate.

In a further embodiment, the compound has the formula



IV

and/or its quinol form, wherein Z is an anion.

Preferably, Z is methanesulfonate.

In another aspect the invention provides a pharmaceutical composition comprising or including a mitochondrially targeted compound comprising a cationic species being a lipophilic cationic moiety covalently coupled to an antioxidant moiety, and an anionic complement for said cationic moiety, wherein

i) the cationic species is capable of mitochondrially targeting the antioxidant species, and

ii) the anionic complement is not HBr, and/or

iii) the anionic complement is non-nucleophilic, and/or

iv) the anionic complement does not exhibit reactivity against the antioxidant moiety.

Conveniently, the anion is selected from the group consisting of alkyl or aryl sulfonates or nitrates.

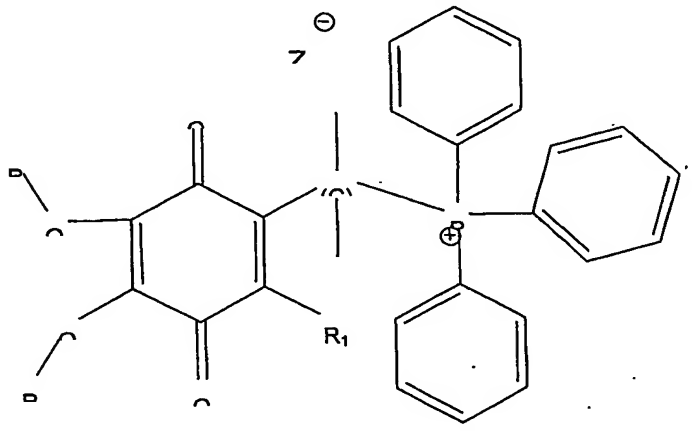
Preferably the anion is methanesulfonate.

The lipophilic cation may be a substituted triphenylphosphonium cation.

Alternatively, the lipophilic cation may be an unsubtituted triphenylphosphonium cation.

Preferably the antioxidant moiety is a quinone or a quinol.

In one embodiment the compound has the general formula III



III

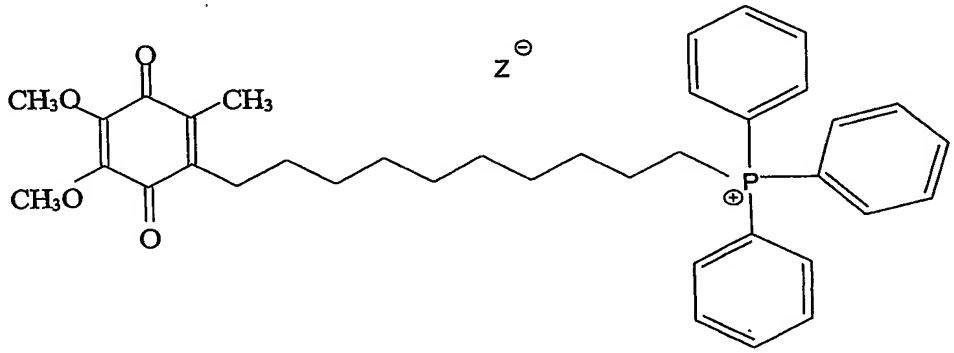
and/or its quinol form, wherein R_1, R_2, and R_3, which can be the same or different, are selected from C_1 to C_5 alkyl (optionally substituted) moieties or H, and wherein n is an integer

from 2 to 10, and wherein each C of the (C)n bridge is saturated, and wherein Z is a non nucleophilic anion.

Preferably Z is selected from the group consisting of alkyl or aryl sulfonates or nitrates.

More preferably Z is methanesulfonate.

In a further embodiment, the compound has the formula



IV

and/or its quinol form, wherein Z is an anion.

Preferably, Z is methanesulfonate.

In a further aspect the present invention consists of a dosage unit comprising or including a mitochondrially targeted compound comprising a cationic species being a lipophilic cationic moiety covalently coupled to an antioxidant moiety, and an anionic complement for said cationic moiety, wherein

i) the cationic species is capable of mitochondrially targeting the antioxidant species, and

ii) the anionic complement is not HBr, and/or

iii) the anionic complement is non-nucleophilic, and/or

iv) the anionic complement does not exhibit reactivity against the antioxidant moiety.

Conveniently, the anion is selected from the group consisting of alkyl or aryl sulfonates or nitrates.

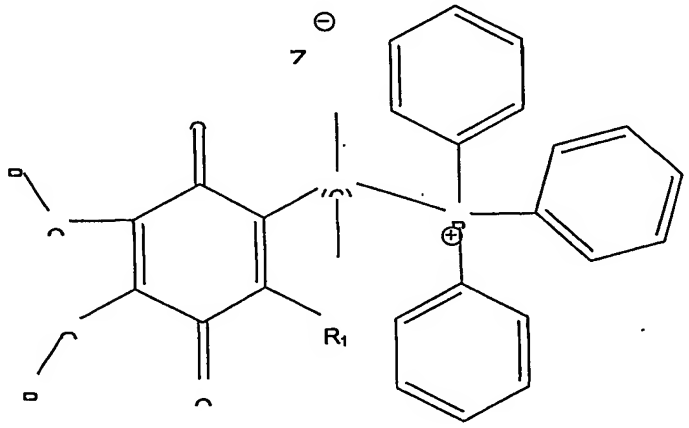
Preferably the anion is methanesulfonate.

The lipophilic cation may be a substituted triphenylphosphonium cation.

Alternatively, the lipophilic cation may be an unsubtituted triphenylphosphonium cation.

Preferably the antioxidant moiety is a quinone or a quinol.

In one embodiment the compound has the general formula III



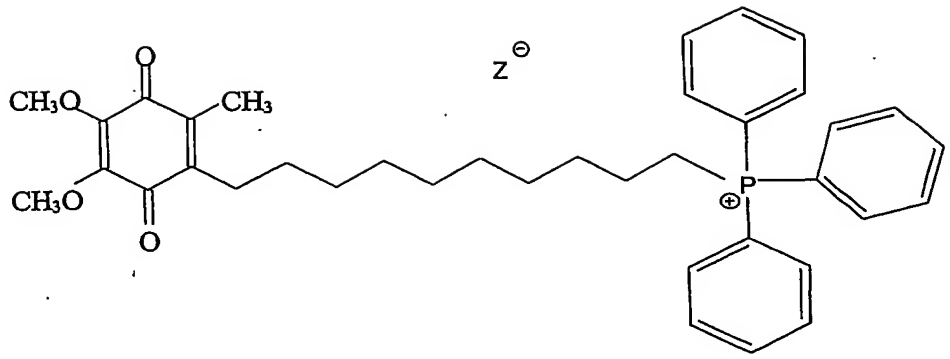
III

and/or its quinol form, wherein R_1, R_2, and R_3, which can be the same or different, are selected from C_1 to C_5 alkyl (optionally substituted) moieties or H, and wherein n is an integer from 2 to 10, and wherein each C of the (C)n bridge is saturated, and wherein Z is a non nucleophilic anion.

Preferably Z is selected from the group consisting of alkyl or aryl sulfonates or nitrates.

More preferably Z is methanesulfonate.

In a further embodiment, the compound has the formula



IV

and/or its quinol form, wherein Z is an anion.

Preferably, Z is methanesulfonate.

In a further aspect, the present invention consists in a stable compound comprising a cationic species being a lipophilic cationic moiety covalently coupled to an antioxidant moiety, and an anionic complement for said cationic moiety, wherein

i) the cationic species is capable of mitochondrially targeting the antioxidant species, and

ii) the anionic complement is not HBr, and/or

iii) the anionic complement is non-nucleophilic, and/or

iv) the anionic complement does not exhibit reactivity against the antioxidant moiety.

Conveniently, the anion is selected from the group consisting of alkyl or aryl sulfonates or nitrates.

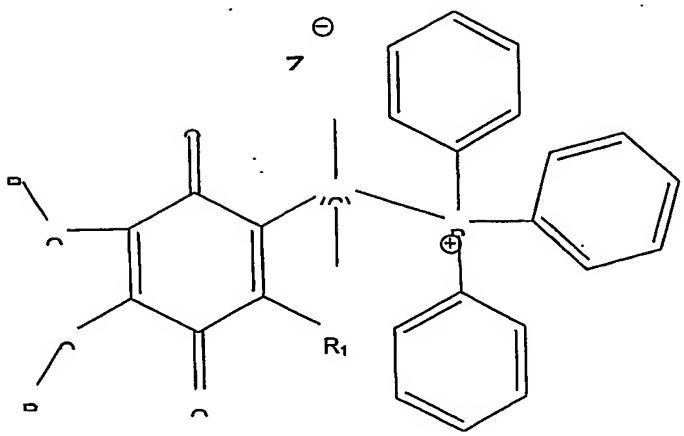
Preferably the anion is methanesulfonate.

The lipophilic cation may be a substituted triphenylphosphonium cation.

Alternatively, the lipophilic cation may be an unsubtituted triphenylphosphonium cation.

Preferably the antioxidant moiety is a quinone or a quinol.

In one embodiment the compound has the general formula III



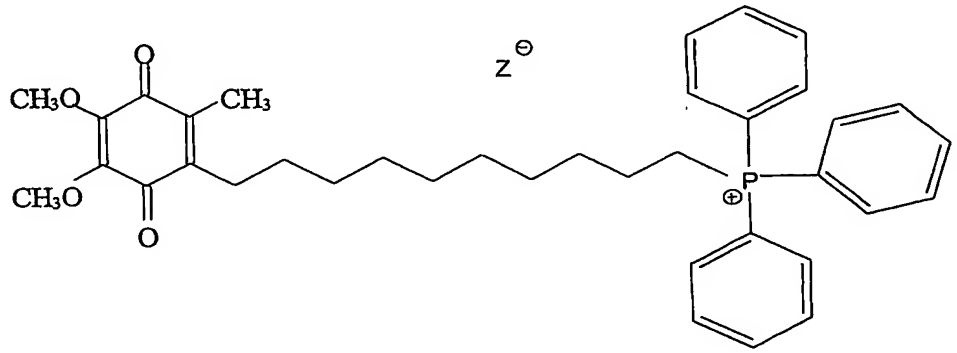
III

and/or its quinol form, wherein R_1, R_2, and R_3, which can be the same or different, are selected from C_1 to C_5 alkyl (optionally substituted) moieties or H, and wherein n is an integer from 2 to 10, and wherein each C of the (C)n bridge is saturated, and wherein Z is a non nucleophilic anion.

Preferably Z is selected from the group consisting of alkyl or aryl sulfonates or nitrates.

More preferably Z is methanesulfonate.

In a further embodiment, the compound has the formula



IV

and/or its quinol form, wherein Z is an anion.

Preferably, Z is methanesulfonate.

In another aspect the invention provides a pharmaceutical composition comprising or including a stable mitochondrially targeted compound comprising a cationic species being a lipophilic cationic moiety covalently coupled to an antioxidant moiety, and an anionic complement for said cationic moiety, wherein

i) the cationic species is capable of mitochondrially targeting the antioxidant species, and

ii) the anionic complement is not HBr, and/or

iii) the anionic complement is non-nucleophilic, and/or

iv) the anionic complement does not exhibit reactivity against the antioxidant moiety.

Conveniently, the anion is selected from the group consisting of alkyl or aryl sulfonates or nitrates.

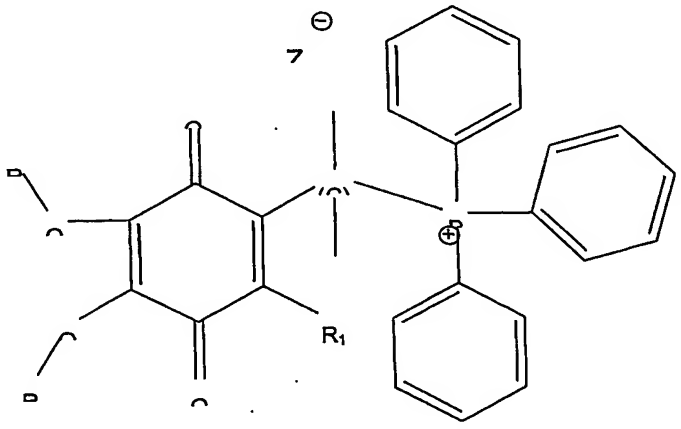
Preferably the anion is methanesulfonate.

The lipophilic cation may be a substituted triphenylphosphonium cation.

Alternatively, the lipophilic cation may be an unsubtituted triphenylphosphonium cation.

Preferably the antioxidant moiety is a quinone or a quinol.

In one embodiment the compound has the general formula III



III

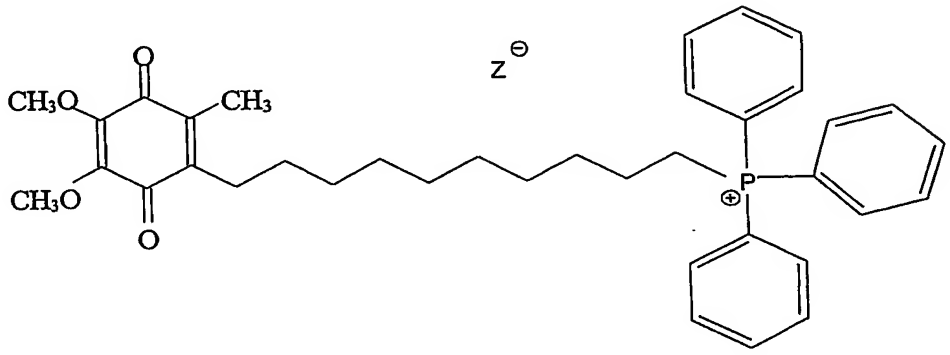
and/or its quinol form, wherein R_1, R_2, and R_3, which can be the same or different, are selected from C_1 to C_5 alkyl (optionally substituted) moieties or H, and wherein n is an integer

from 2 to 10, and wherein each C of the (C)n bridge is saturated, and wherein Z is a non nucleophilic anion.

Preferably Z is selected from the group consisting of alkyl or aryl sulfonates or nitrates.

More preferably Z is methanesulfonate.

In a further embodiment, the compound has the formula



IV

and/or its quinol form, wherein Z is an anion.

Preferably, Z is methanesulfonate.

In a further aspect the present invention consists of a dosage unit comprising or including a stable mitochondrially targeted compound comprising a cationic species being a lipophilic cationic moiety covalently coupled to an antioxidant moiety, and an anionic complement for said cationic moiety, wherein

i) the cationic species is capable of mitochondrially targeting the antioxidant species, and

ii) the anionic complement is not HBr, and/or

iii) the anionic complement is non-nucleophilic, and/or

iv) the anionic complement does not exhibit reactivity against the antioxidant moiety.

Conveniently, the anion is selected from the group consisting of alkyl or aryl sulfonates or nitrates.

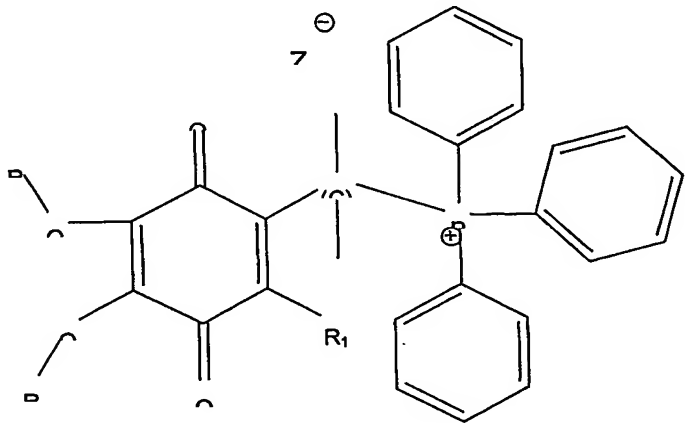
Preferably the anion is methanesulfonate.

The lipophilic cation may be a substituted triphenylphosphonium cation.

Alternatively, the lipophilic cation may be an unsubtituted triphenylphosphonium cation.

Preferably the antioxidant moiety is a quinone or a quinol.

In one embodiment the compound has the general formula III



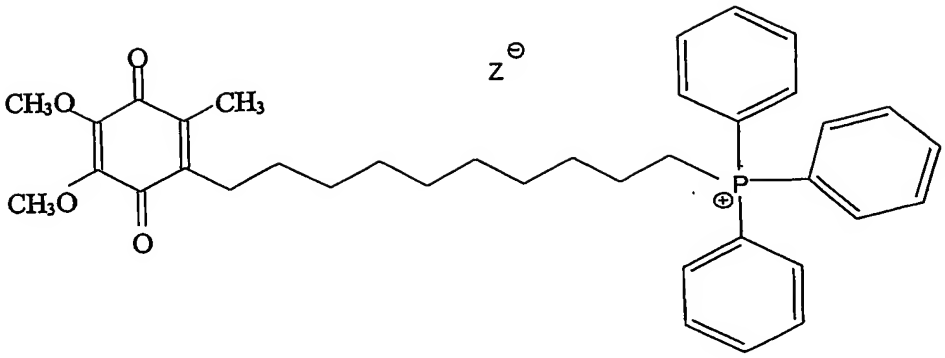
III

and/or its quinol form, wherein R_1, R_2, and R_3, which can be the same or different, are selected from C_1 to C_5 alkyl (optionally substituted) moieties or H, and wherein n is an integer from 2 to 10, and wherein each C of the (C)n bridge is saturated, and wherein Z is a non nucleophilic anion.

Preferably Z is selected from the group consisting of alkyl or aryl sulfonates or nitrates.

More preferably Z is methanesulfonate.

In a further embodiment, the compound has the formula



IV

and/or its quinol form, wherein Z is an anion.

Preferably, Z is methanesulfonate.

In another aspect, the invention provides **a mitochondrially-targeted antioxidant compound** which comprises a lipophilic cation covalently coupled to an antioxidant moiety, **wherein**

i) the compound is crystalline or solid, and/or

ii) the compound has a partition coefficient (octanol:water) less than about 20, and/or

iii) the compound is a salt form in which the anion exhibits no reactivity against the antioxidant moiety, and/or

iv) the compound is in a salt form where the salt is acceptable for pharmaceutical preparation, and/or

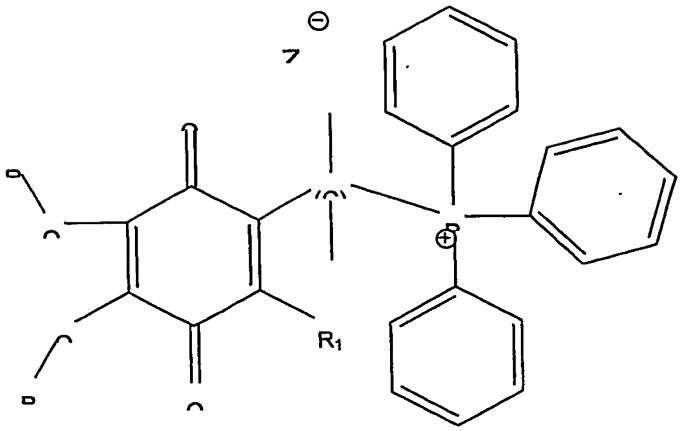
v) the compound is a salt form in which the anion is non nucleophilic.

Conveniently, an acceptable salt form is that of the methanesulfonate.

Conveniently, the lipophilic cation (preferably unsubstituted) is the triphenylphosphonium cation.

Conveniently, the antioxidant moiety is a quinone or a quinol.

In a second aspect the present invention consists in a **mitochondrially targeted antioxidant compound** of the general formula I



I

wherein R_1, R_2, and R_3, which can be the same or different, are selected from C_1 to C_5 alkyl (optionally substituted) moieties or H, and wherein n is an integer from 2 to 10, and wherein each C of the (C)n bridge is saturated, and wherein Z is an anion, and wherein

i) the compound is crystalline or solid, and/or

ii) the compound has a partition coefficient (octanol:water) less than about 20, and/or

iii) the compound is a salt form in which the anion exhibits no reactivity against the antioxidant moiety, and/or

iv) the compound is in a salt form where the salt is acceptable for pharmaceutical preparation, and/or

v) Z is non nucleophilic.

Conveniently, an acceptable salt form is that of the methanesulfonate.

Conveniently, the lipophilic cation (preferably unsubstituted) is the triphenylphosphonium cation.

Conveniently, the antioxidant moiety is a quinone or a quinol.

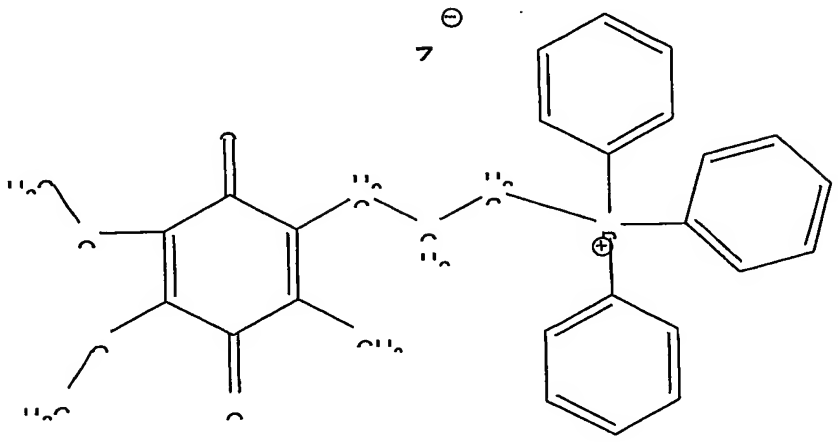
In a further aspect, the present invention consists in a **dosage unit** suitable for oral administration comprising as an active ingredient a compound in accordance with the present invention, the compound being of a crystalline form and/or non-liquid form.

Preferably said compound is in the form of a salt.

Preferably said compound has a partition coefficient less than about 10.

Preferably the compound has a bridge of 3 carbon atoms (C_3).

In a further aspect, the invention provides a mitochondrially-targeted antioxidant compound of the general formula (2)



(2)

wherein Z is an anion that is not nucleophilic and/or does not exhibit reactivity against the antioxidant moiety, for example but not exclusively alkyl or aryl sulfonates or nitrates.

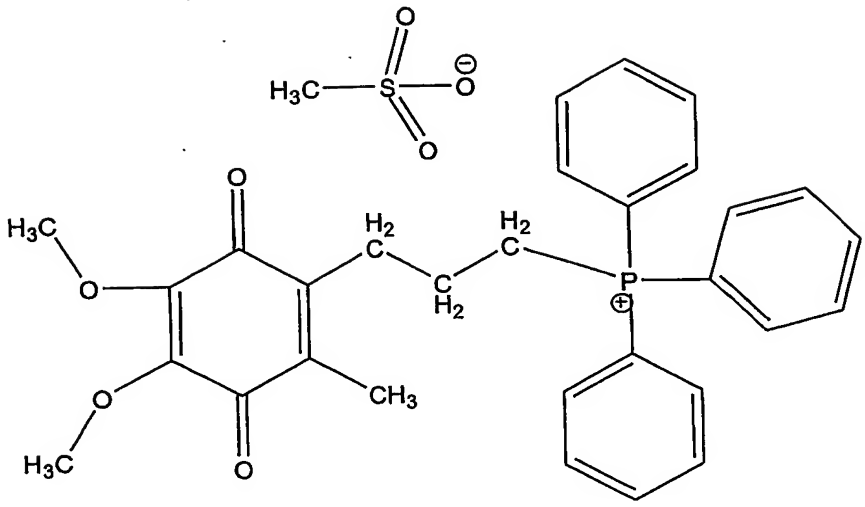
Preferably, Z is a pharmaceutically acceptable anion.

More preferably, Z is an alkyl or aryl sulfonate.

Preferably said compound is in the form of a salt.

Preferably said compound has a partition coefficient (octanol:water) less than about 20, more preferably less than about 10.

Most preferably, the mitochondrially-targeted antioxidant has the formula (2)



(2)

In a further aspect, the present invention provides a **pharmaceutical composition** suitable for treatment of a patient who would benefit from reduced oxidative stress which comprises or includes an effective amount of a mitochondrially-targeted antioxidant compound of the present invention in combination with one or more pharmaceutically acceptable carriers or diluents.

Preferably the mitochondrially targeted antioxidant is a compound of formula I.

More preferably the mitochondrially targeted antioxidant is compound (2).

In a further aspect, the invention provides **a method of reducing oxidative stress in a cell** which comprises the step of administering to said cell a mitochondrially targeted antioxidant compound as defined above.

Preferably the mitochondrially targeted antioxidant is a compound of formula I.

More preferably the mitochondrially targeted antioxidant is compound (2) .

In still a further aspect, the invention provides **a method of therapy or prophylaxis of a patient** who would benefit from reduced oxidative stress which comprises or includes the step of administering to said patient a mitochondrially-targeted antioxidant compound as defined above.

Preferably the mitochondrially targeted antioxidant is a compound of formula I.

More preferably the mitochondrially targeted antioxidant is compound (2) .

In a further aspect the invention provides a **pharmaceutical composition** suitable for the treatment of a patient who would benefit from reduced oxidative stress, which comprises an effective amount of a mitochondrially-targeted antioxidant compound comprising a lipophilic cation covalently coupled to an antioxidant moiety in combination with one or more pharmaceutically acceptable carriers or diluents, and wherein

i) the compound is crystalline or solid, and/or

ii) the compound has a partition coefficient (octanol:water) less than about 20, and/or

iii) the compound is a salt form in which the anion exhibits no reactivity against the antioxidant moiety, and/or

iv) the compound is in a salt form where the salt is acceptable for pharmaceutical preparation, and/or

v) the compound is a salt form in which the anion is non nucleophilic.

Conveniently, an acceptable salt form is that of the methanesulfonate.

Conveniently, the lipophilic cation (preferably unsubstituted) is the triphenylphosphonium cation.

Conveniently, the antioxidant moiety is a quinone or a quinol.

Preferably the mitochondrially targeted antioxidant is a compound of formula I.

More preferably the mitochondrially targeted antioxidant is compound **(2)** .

In another aspect the invention provides **a pharmaceutical composition** suitable for the treatment of a patient who would benefit from reduced oxidative stress, or reduced symptoms of ageing, which comprises an effective amount of a mitochondrially targeted antioxidant compound as previously described in combination with one or more pharmaceutically acceptable carriers or diluents, and wherein

i) the compound is crystalline or solid, and/or

ii) the compound has a partition coefficient (octanol:water) less than about 20, and/or

iii) the compound is a salt form in which the anion exhibits no reactivity against the antioxidant moiety, and/or

iv) the compound is in a salt form where the salt is acceptable for pharmaceutical preparation, and/or

v) the compound is a salt form in which the anion is non nucleophilic.

Conveniently, an acceptable salt form is that of the methanesulfonate.

Conveniently, the lipophilic cation (preferably unsubstituted) is the triphenylphosphonium cation.

Conveniently, the antioxidant moiety is a quinone or a quinol.

Preferably the mitochondrially targeted antioxidant is a compound of formula I.

More preferably the mitochondrially targeted antioxidant is compound (2).

In another aspect the invention provides a **pharmaceutical composition** suitable for the treatment of a patient who would benefit from reduced symptoms of aging, which comprises an effective amount of a mitochondrially-targeted antioxidant compound comprising a lipophilic cation covalently coupled to an antioxidant moiety in combination with one or more pharmaceutically acceptable carriers or diluents, and wherein

i) the compound is crystalline or solid, and/or

ii) the compound has a partition coefficient (octanol:water) less than about 20, and/or

iii) the compound is a salt form in which the anion exhibits no reactivity against the antioxidant moiety, and/or

iv) the compound is in a salt form where the salt is acceptable for pharmaceutical preparation, and/or

v) the compound is a salt form in which the anion is non nucleophilic.

Conveniently, an acceptable salt form is that of the methanesulfonate.

Conveniently, the lipophilic cation (preferably unsubstituted) is the triphenylphosphonium cation.

Conveniently, the antioxidant moiety is a quinone or a quinol.

Preferably the mitochondrially targeted antioxidant is a compound of formula I.

More preferably the mitochondrially targeted antioxidant is compound (2) .

In another aspect the invention provides **a method of therapy or prophylaxis of a patient** who would benefit from reduced oxidative stress, or reduced symptoms of ageing which comprises the step of administering to the patient, a mitochondrially-targeted antioxidant compound comprising a liphohilic cation covalently coupled to an antioxidant moiety, and **wherein**

i) the compound is crystalline or solid, and/or

ii) the compound has a partition coefficient (octanol:water) less than about 20, and/or

iii) the compound is a salt form in which the anion exhibits no reactivity against the antioxidant moiety, and/or

iv) the compound is in a salt form where the salt is acceptable for pharmaceutical preparation, and/or

v) the compound is a salt form in which the anion is non nucleophilic.

Conveniently, an acceptable salt form is that of the methanesulfonate.

Conveniently, the lipophilic cation (preferably unsubstituted) is the triphenylphosphonium cation.

Conveniently, the antioxidant moiety is a quinone or a quinol.

Preferably the mitochondrially targeted antioxidant is a compound of formula I.

More preferably the mitochondrially targeted antioxidant is compound **(2)** .

In another aspect the invention provides a **method of therapy or prophylaxis** of a patient who would benefit from reduced oxidative stress, or reduced symptoms of ageing, which comprises the step of administering to the patient a mitochondrially-targeted antioxidant compound as previously described.

In another aspect the invention provides **a method of reducing oxidative stress in a cell,** which comprises the step of administering to the cell a mitochondrially-targeted antioxidant compound comprising a lipophilic cation covalently coupled to an antioxidant moiety, **wherein**

i) the compound is crystalline or solid, and/or

ii) the compound has a partition coefficient (octanol:water) less than about 20, and/or

iii) the compound is a salt form in which the anion exhibits no reactivity against the antioxidant moiety, and/or

iv) the compound is in a salt form where the salt is acceptable for pharmaceutical preparation, and/or

v) the compound is a salt form in which the anion is non nucleophilic.

Conveniently, an acceptable salt form is that of the methanesulfonate.

Conveniently, the lipophilic cation (preferably unsubstituted) is the triphenylphosphonium cation.

Conveniently, the antioxidant moiety is a quinone or a quinol.

Preferably the mitochondrially targeted antioxidant is a compound of formula I.

More preferably the mitochondrially targeted antioxidant is compound **(2)**.

In another aspect the invention provides **a method of reducing oxidative stress in a cell,** which comprises the step of administering to the cell a mitochondrially-targeted antioxidant as previously described.

Preferably the mitochondrially targeted antioxidant is a compound of formula I.

More preferably the mitochondrially targeted antioxidant is compound **(2)**.

In another aspect the invention provides **the use of a mitochondrially-targeted antioxidant compound** comprising a lipophilic cation covalently coupled to an antioxidant moiety in the preparation or manufacture with other material or materials of a medicament, dosage unit, or pharmaceutical composition effective for use in the reduction of oxidative stress in a patient, **wherein**

i) the compound is crystalline or solid, and/or

ii) the compound has a partition coefficient (octanol:water) less than about 20, and/or

iii) the compound is a salt form in which the anion exhibits no reactivity against the antioxidant moiety, and/or

iv) the compound is in a salt form where the salt is acceptable for pharmaceutical preparation, and/or

v) the compound is a salt form in which the anion is non nucleophilic.

Conveniently, an acceptable salt form is that of the methanesulfonate.

Conveniently, the lipophilic cation (preferably unsubstituted) is the triphenylphosphonium cation.

Conveniently, the antioxidant moiety is a quinone or a quinol.

Preferably the mitochondrially targeted antioxidant is a compound of formula I.

More preferably the mitochondrially targeted antioxidant is compound **(2)**.

In another aspect the invention provides **the use of a compound as previously described** in the preparation or manufacture with other material or materials of a medicament, dosage unit, or pharmaceutical composition effective for use in for the reduction of oxidative stress in a patient.

In another aspect the invention provides **the use of a mitochondrially-targeted antioxidant compound** comprising a lipophilic cation covalently coupled to an antioxidant in the preparation or manufacture with other material or materials of a medicament, dosage unit, or pharmaceutical composition effective for use in moiety for the reduction of symptoms of aging in a patient, **wherein**

i) the compound is crystalline or solid, and/or

ii) the compound has a partition coefficient (octanol:water) less than about 20, and/or

iii) the compound is a salt form in which the anion exhibits no reactivity against the antioxidant moiety, and/or

iv) the compound is in a salt form where the salt is acceptable for pharmaceutical preparation, and/or

v) the compound is a salt form in which the anion is non nucleophilic.

Conveniently, an acceptable salt form is that of the methanesulfonate.

Conveniently, the lipophilic cation (preferably unsubstituted) is the triphenylphosphonium cation.

Conveniently, the antioxidant moiety is a quinone or a quinol.

In another aspect the invention provides **the use of a compound as previously described** in the preparation or manufacture with other material or materials of a medicament, dosage unit, or pharmaceutical composition effective for use for the reduction of symptoms of aging in a patient.

In another aspect the invention provides **the use of a mitochondrially-targeted antioxidant compound** comprising a lipophilic cation covalently coupled to an antioxidant moiety in the preparation or manufacture with other material or materials of a medicament, dosage unit, or pharmaceutical composition effective for use for the reduction of oxidative stress in a cell, **wherein**

i) the compound is crystalline or solid, and/or

ii) the compound has a partition coefficient (octanol:water) less than about 20, and/or

iii) the compound is a salt form in which the anion exhibits no reactivity against the antioxidant moiety, and/or

iv) the compound is in a salt form where the salt is acceptable for pharmaceutical preparation, and/or

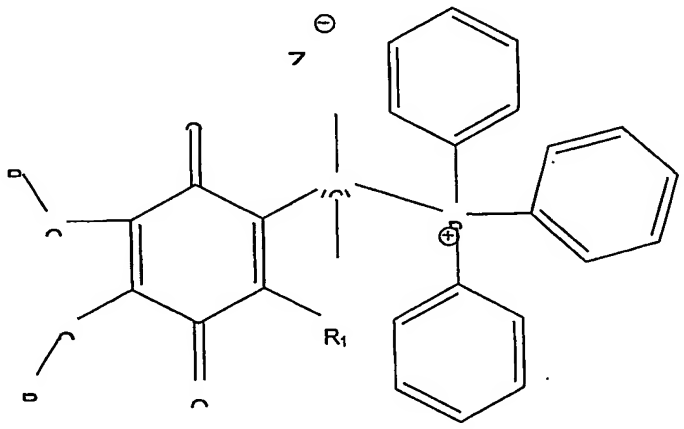
v) the compound is a salt form in which the anion is non nucleophilic.

Conveniently, an acceptable salt form is that of the methanesulfonate.

Conveniently, the lipophilic cation (preferably unsubstituted) is the triphenylphosphonium cation.

Conveniently, the antioxidant moiety is a quinone or a quinol.

In another aspect the invention provides a **composition** comprising or including a compound of the general formula I



I

herein it is crystalline in form and has a partition coefficient herein defined less than about 20, and/or the compound of the general formula is crystalline in form.

In another aspect the present invention provides **a dosage unit** comprising a mitochondrially-targeted antioxidant compound, **wherein**

i) the compound is crystalline or solid, and/or

ii) the compound has a partition coefficient (octanol:water) less than about 20, and/or

iii) the compound is a salt form in which the anion exhibits no reactivity against the antioxidant moiety, and/or

iv) the compound is in a salt form where the salt is acceptable for pharmaceutical preparation, and/or

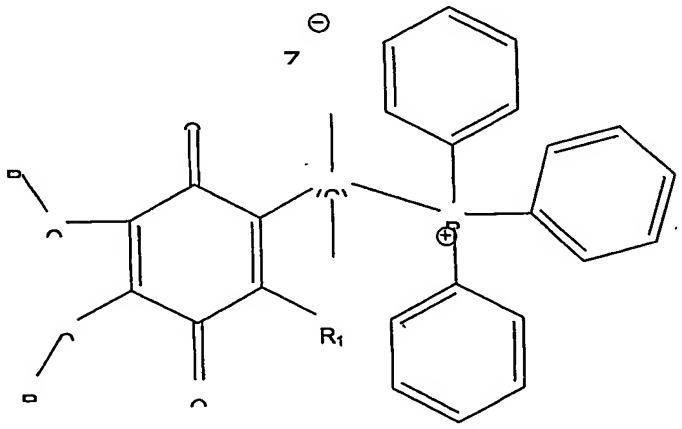
v) the compound is a salt form in which the anion is non nucleophilic, and

wherein the unit can be a capsule such as a gelatinized capsule, a tablet, or any other orally-administerable dosage unit.

Preferably an effective amount is present in any such dosage unit such effective amount being related to the dosage regime for the taking of such dosage units for any of the indications herein described.

In another aspect the invention provides the **use of a compound as previously described in the preparation or manufacture** with other material or materials **of a medicament, dosage unit, or pharmaceutical composition** effective for use in the reduction of oxidative stress in a cell.

In yet another aspect the present invention consists in a **method of synthesis** of a compound with a moiety or the moiety of the formula I



I

(and/or its quinone form) wherein R_1, R_2, and R_3, which can be the same or different, are selected from C_1 to C_3 alkyl (optionally substituted) moieties, and wherein n is an integer from 2 to 10, and wherein each C of the (C)n bridge is saturated, and **wherein**

i) the compound is crystalline or solid, and/or

ii) the compound has a partition coefficient (octanol:water) less than about 20, and/or

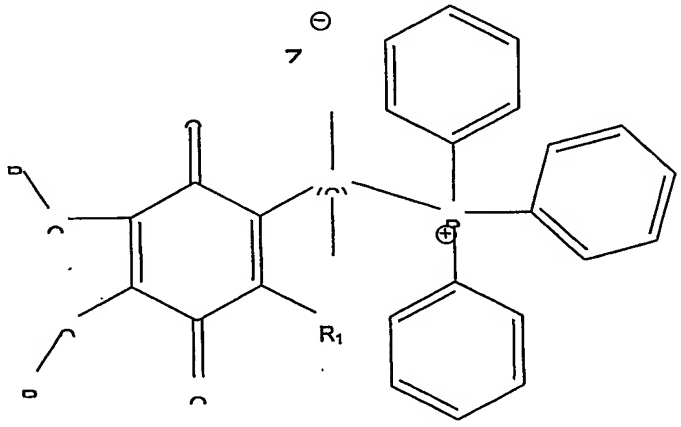
iii) the compound is a salt form in which the anion exhibits no reactivity against the antioxidant moiety, and/or

iv) the compound is in a salt form where the salt is acceptable for pharmaceutical preparation, and/or

v) the compound is a salt form in which the anion is non nucleophilic.

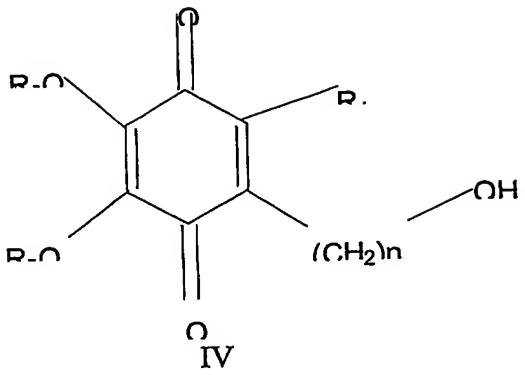
Preferably the formation of the compound from triphenylphosphonium does not involve reaction solvent.

In yet another aspect the present invention consists in **a method of synthesis** of a compound with a moiety or the moiety of the formula I



I

(and/or its quinone form) wherein R_1, R_2, and R_3, which can be the same or different, are selected from C_1 to C_3 alkyl (optionally substituted) moieties, and wherein n is an integer from 2 to 10, and wherein each C of the (C)n bridge is saturated, which comprises or includes the reaction of a compound of the formula IV



IV

(and/or its quinol form) in the presence of Ph_3PHX and Ph_3P, where X is a halogen atom.

Preferably X is preferably bromine, iodine or chlorine (most preferably bromine).

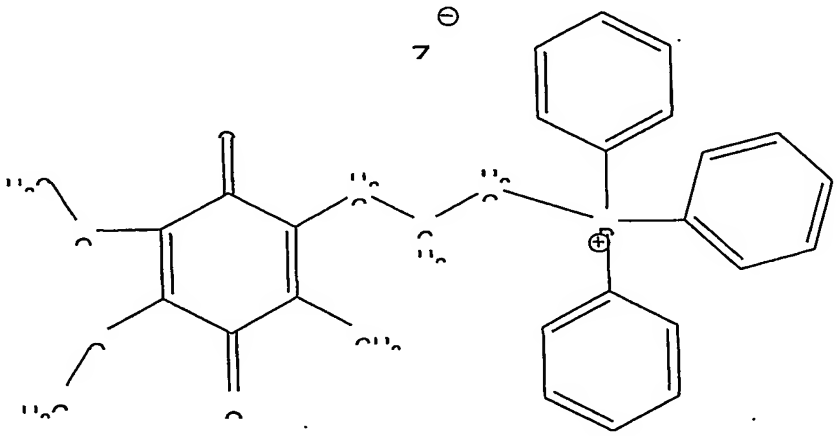
Preferably n is 2 to 5.

More preferably n is 3.

Preferably the reaction is maintained as a temperature below which significant amounts of R_2PPh_3, or R_3PPh_3, are not formed by ether cleavage, eg; the mixture is preferably kept below 80°C.

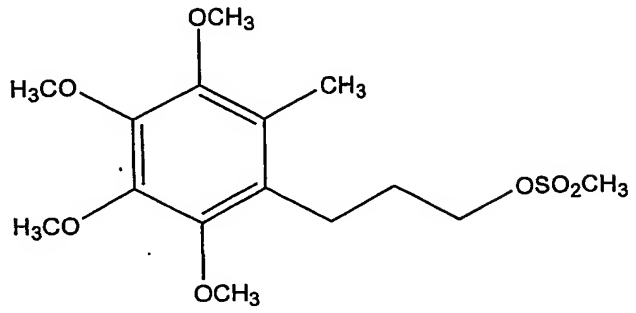
Preferably the formation of the compound from triphenylphosphonium does not involve reaction solvent.

In yet another aspect the present invention consists in **a method of synthesis** of a compound with a moiety or the moiety of the formula (2)



(2)

(and/or its quinol form), wherein Z is an anion, which comprises or includes the reaction of a compound of the formula (3)



(3)

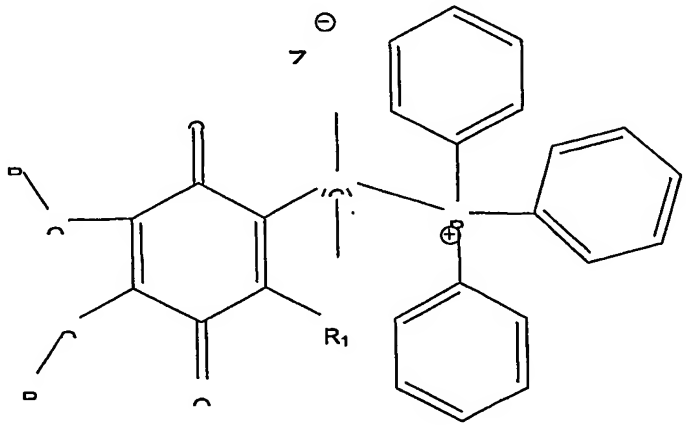
in the presence of Ph_3P and X, where X comprises or includes a halogen atom.

Preferably X comprises or includes bromine, iodine or chlorine (most preferably iodine).

Preferably the reaction is maintained as a temperature below which significant amounts of $MePPh_3$ are not formed by ether cleavage, eg; the mixture is preferably kept below 80°C.

Preferably the formation of the compound from triphenylphosphonium does not involve reaction solvent.

In a further aspect the present invention consists in **a method of synthesis** of a compound with a moiety or the moiety of the formula I



I

(and/or its quinone form) wherein R_1, R_2, and R_3, which can be the same or different, are selected from C_1 to C_3 alkyl (optionally substituted) moieties, and wherein n is an integer from 2 to 10, and wherein each C of the (C)n bridge is saturated, said method substantially as herein described.

Preferably, said method is reliant upon the method depicted in Scheme 1 herein.

More preferably, said method is reliant upon the method depicted in Scheme 1 herein in conjunction with that depicted in Scheme 3 herein.

Preferably the formation of the compound from triphenylphosphonium does not involve reaction solvent.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed before the priority date of each claim of this application.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

Throughout this specification the term "quinone", whether used alone or prefixed with another term to describe the oxidized form of a compound, will be understood to include within its scope the reduced form of that compound, that is, the quinol form. Similarly, reference to a quinone, by structural depiction for example, also includes within its scope the quinol form.

Throughout this specification the term "quinol", whether used alone or prefixed with another term to describe the reduced form of a compound, will be understood to include within its scope the oxidised form of that compound, that is, the quinone form. Similarly, reference to a quinol, by structural depiction for example, also includes within its scope the quinone form.

As used herein the term "and/or" includes both "and" and "or" as options.

As used herein, the term "partition coefficient" and "partition coefficient (octanol:water)" refer to the octan-1-ol/phosphate buffered saline partition coefficient determined at 25°C or 37°C (see Kelso, G.F., Porteous, C.M., Coulter, C.V., Hughes, G. Porteous, W.K., Ledgerwood, E.C., Smith, R.A.J. and Murphy, M.P. 2001 J Biol Chem 276 4588. Smith,

R.A.J., Porteous, C.M., Coulter, C.V. and Murphy, M.P. 1999 Eu. J Biochem 263, 709. Smith, R.A.J., Porteous, C.M., Gane, A.M. and Murphy, M.P. 2003 Proc Nat Acad Sci 100, 5407.), or the octanol/water partition coefficient calculated using Advanced Chemistry Development (ACD) Software Solaris V4.67 as described in Jauslin, M. L., Wirth, T., Meier, T., and Schoumacher, F., 2002, Hum Mol Genet 11, 3055.

As used herein, the phrase "acceptable for pharmaceutical preparation" includes within its meaning not only an acceptability with regard to pharmaceutical administration, but also in respect of formulation for, for example, acceptable stability, shelf life, hygroscopicity, preparation and the like.

Although broadly as defined above, the invention is not limited thereto but also consists of embodiments of which the following description provides examples.

In particular, a better understanding of the invention will be gained with reference to the accompanying drawings, in which:

Figure 1 is a graph which shows the uptake by isolated mitochondria of compound **4**, a mitochondrially-targeted antioxidant according to the present invention;

Figure 2 is a graph which shows the accumulation of compound **4** by isolated mitochondria;

Figure 3 is a graph which shows a comparison of a compound **4** uptake with that of the triphenylphosphonium cation (TPMP);

Figure 4 is a graph which shows that compound **4** protects mitochondria against oxidative damage;

Figure 5 is a graph which compares compound **4** with vitamin E and the effect of uncoupler and other lipophilic cations;

Figure 6 is a graph which shows that compound **4** protects mitochondrial function from oxidative damage;

Figure 7 is a graph which shows the effect of compound **4** on mitochondrial function;

Figure 8 is a graph which shows the uptake of compound **4** by cells;

Figure 9 is a graph which shows the energisation-sensitive uptake of compound **4** by cells;

Figure 10 is a graph which shows the effect of compound **4** on cell viability;

Figure 11 is a graph which shows the UV absorption spectra of mitoquinone **(1)** and mitoquinol;

Figure 12 presents graphs showing the reduction of mitoquinone (**1**) and the oxidation of mitoquinol by complexes of the respiratory chain;

Figure 13 presents graphs showing mitoquinone (**1**) can accept electrons from complex I and complex II of the respiratory chain;

Figure 14 presents graphs showing mitoquinone (**1**) can accept electrons within intact mitochondria;

Figure 15 is a graph which shows the accumulation of mitoquinol within mitochondria;

Figure 16 presents graphs showing the effect of mitoquinol on mitochondrial function; and

Figure 17 is a graph which shows that compounds **1** and **2** protect mitochondria against oxidative damage.

Figure 18 presents graph showing the effect of compounds 1 and 4 on sinus coronary flow.

Figure 19 presents a graph showing the effect of compound **1** and compound **2** on left ventricular diastolic pressure.

Figure 20 present a graph which shows the effect of compound **1** and compound **2** on heart rate.

Figure 21 presents graphs showing the rate of left ventricular change.

Figure 22 depicts graphs showing the effect of compound **1** and compound **2** on post ischaemia mitochondrially respiratory function.

Figure 23 is a graph depicting the stability of pure compound **1** (batch no.3) in clear glass bottles at 40°C, 75%RH; 25°C, 50%RH and 5°C over silica gel.

Figure 24 is a graph depicting the stability of Compound **1** (batch no. 4) at 25°C, 50%RH.

Figure 25 is a graph depicting the stability of compound **1**: β–cyclodextrin complex (1:1) at 4°C over silica, 25°C, 50%RH and 40°C, 75%RH.

Figure 26 is a graph depicting the stability of compound **1**: β–cyclodextrin complex (1:2) at 4°C over silica, 25°C, 50%RH and 40°C, 75%RH.

Figure 27 is a graph depicting the stability of compound **1**: β–cyclodextrin complex (1:4) at 4°C over silica, 25°C, 50%RH and 40°C, 75%RH.

As stated above, the focus of this invention is on the mitochondrial targeting of compounds, primarily for the purpose of therapy and/or prophylaxis to reduce oxidative stress.

Mitochondria have a substantial membrane potential of up to 180 mV across their inner membrane (negative inside). Because of this potential, membrane permeant, lipophilic cations accumulate several-hundred fold within the mitochondrial matrix.

The applicants have now found that by covalently coupling lipophilic cations (preferably the lipophilic triphenylphosphonium cation) to an antioxidant the compound can be delivered to the mitochondrial matrix within intact cells. The antioxidant is then targeted to a primary production site of free radicals and reactive oxygen species within the cell, rather than being randomly dispersed.

In principle, any lipophilic cation and any antioxidant capable of being transported through the mitochondrial membrane and accumulated within the mitochondria of intact cells, can be employed in forming the compounds of the invention. It is however preferred that the lipophilic cation be the triphenylphosphonium cation herein exemplified, and that the lipophilic cation is linked to the antioxidant moiety by a carbon chain having 1 to about 30 carbon atoms, for example 1 to about 20, 1 to about 10, or 1 to about 6 carbon atoms.

Conveniently the carbon chain is an alkylene group (for example, C_1-C_{20}, or C_1-C_{15}), yet carbon chains which optionally include one or more double or triple bonds are also within the scope of the invention. Also included are carbon chains which include one or more substituents (such as hydroxyl, carboxylic acid or amide groups), and/or include one or more side chains or branches, selected from unsubstituted or substituted alkyl alkenyl or alkynyl groups.

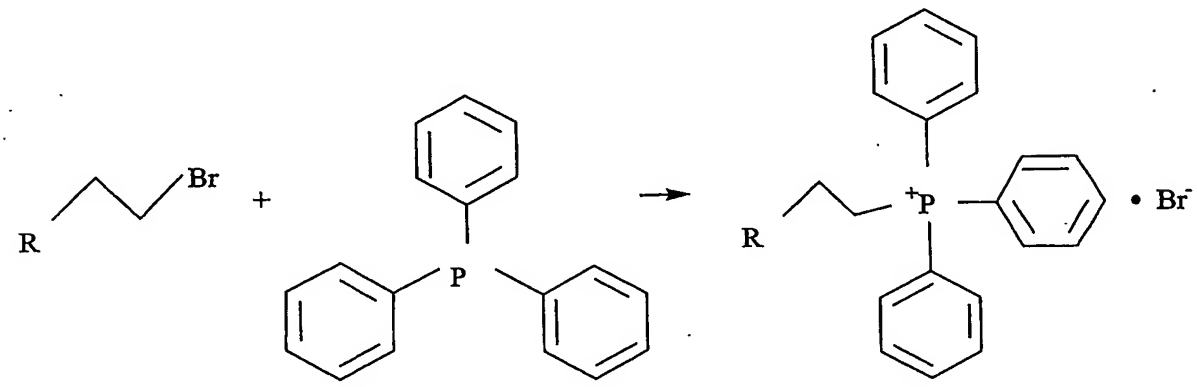
In one embodiment, the lipophilic cation is linked to the antioxidant moiety by a straight chain alkylene group having 1 to 10 carbon atoms; such as, for example a propylene group.

It will be appreciated by those skilled in the art that moieties other than a straight alkylene may be used to covalently couple the antioxidant moiety to the lipophilic cation, for example, substituted or branched alkyl groups, peptide bonds, and the like.

In some embodiments, the linking group is an ethylene, propylene, butylene, pentylene or decylene group.

Other lipophilic cations which may covalently be coupled to antioxidants in accordance with the present invention include the tribenzyl ammonium and phosphonium cations.

Such preferred antioxidant compounds, including those of general formulae I and II herein, can be readily prepared, for example, by the following reaction:



The general synthesis strategy is to heat a precursor containing a suitable leaving group, preferably a alkyl sulfonyl, bromo or iodo precursor with greater than 1 equivalents of triphenylphosphine under argon for several days. The phosphonium compound is then isolated as its salt. To do this the product is triturated repeatedly with diethyl ether until an off-white solid remains. This is then dissolved in chloroform or dichloromethane and precipitated with diethyl ether to remove the excess triphenylphosphine. This is repeated until the solid no longer dissolves in chloroform. At this point the product is recrystallised several times from a suitable solvent such as chloroform, acetone, ethyl acetate or higher alcohols.

It will also be appreciated that the anion of the antioxidant compound thus prepared can readily be exchanged with another pharmaceutically or pharmacologically acceptable anion, if this is desirable or necessary, using ion exchange or other techniques known in the art.

The applicants have determined that the stability of the salt form of the antioxidant compound is enhanced when the anion is not nucleophilic. It is also desirable that the anion is a pharmaceutically acceptable anion.

Examples of non-nucleophilic anions include hexafluoroantimonate, -arsenate or -phosphate, or tetraphenylborate, tetra(perfluorophenyl)borate or other tetrafluoroborates,

trifluoromethane sulfonate, aryl and alkyl sulfonates such as methanesulfonate and p-toluenesulfonate, and phosphates.

Examples of pharmaceutically acceptable anions include halogen ions such as a fluoride ion, chloride ion, bromide ion and iodide ion; anions of inorganic acid salts such as nitrate, perchlorate, sulfate, phosphate, and carbonate; pharmaceutically acceptable anions of lower alkylsulfonic acid salts such as methanesulfonic acid, and ethanesulfonic acid salts; pharmaceutically acceptable anions of arylsulfonic acid salts such as benzenesulfonic acid, 2-naphthalenesulfonic acid and p-toluenesulfonic acid salts; pharmaceutically acceptable anions of organic acid salts such as trichloroacetic acid, trifluoroacetic acid, hydroxyacetic acid, benzoic acid, mandelic acid, butyric acid, propionic acid, formic acid, fumaric acid, succinic acid, citric acid, tartaric acid, oxalic acid, maleic acid, acetic acid, malic acid, lactic acid, and ascorbic acid salts; and pharmaceutically acceptable anions of acidic amino acid salts such as glutamic acid and asparatic acid salts.

In the case of preferred antioxidant compounds of the invention, the halogen anion precursor is exchanged for aryl or alkyl sulphonate anions. Examples include, but are not limited to, benzene sulfonate, p-toluene sulfonate, 2-napthylene sulphonate, methanesulfonate, ethanesulfonate, propanesulfonate. A particularly preferred anion is the methanesulfonate anion.

The same general procedure can be used to make a wide range of mitochondrially targeted compounds with different antioxidant moieties R attached to the triphenylphosphonium (or other lipophilic cationic) salt. These will include a series of vitamin E derivatives, in which the length of the bridge linking the Vitamin-E function with the triphenylphosphonium salt is varied. Other antioxidants which can be used as R include chain breaking antioxidants, such as butylated hydroxyanisole, butylated hydroxytoluene, quinols and general radical scavengers such as derivatised fullerenes. In addition, spin traps, which react with free radicals to generate stable free radicals can also be synthesized. These will include derivatives of 5,5-dimethylpyrroline-*N*-oxide, *tert*-butylnitrosobenzene, *tert*-nitrosobenzene, α-phenyl-*tert*-butylnitrone and related compounds.

In some embodiments of the invention, the antioxidant compound is a quinol derivative of the formula II defined above. For example, a quinol derivative of the invention is the compound mitoquinol (compound 1) as defined above. A further example of a compound of the invention is a compound of formula II in which $(C)_n$ is $(CH_2)_5$, and the quinol moiety is the same as that of mitoquinol. Yet a further example of a compound of the

invention is a compound of formula II in which $(C)_n$ is $(CH_2)_3$, and the quinol moiety is the same as that of mitoquinol, which is referred to herein as compound 2.

Once prepared, the antioxidant compound of the invention, in any pharmaceutically appropriate form and optionally including pharmaceutically-acceptable carriers or additives, will be administered to the patient requiring therapy and/or prophylaxis. Once administered, the compound will target the mitochondria within the cell.

Pharmaceutically acceptable carriers or additives may be chosen so as to enhance the stability of the antioxidant compound, or to enhance its bioavailability.

For example, carrier molecules such as cyclodextrin and derivatives thereof are well known in the art for their potential as complexation agents capable of altering the physicochemical attributes of drug molecules. For example, cyclodextrins may stabilize (both thermally and oxidatively), reduce the volatility of, and alter the solubility of, active agents with which they are complexed. Cyclodextrins are cyclic molecules composed of glucopyranose ring units which form toroidal structures. The interior of the cyclodextrin molecule is hydrophobic and the exterior is hydrophilic, making the cyclodextrin molecule water soluble. The degree of solubility can be altered through substitution of the hydroxyl groups on the exterior of the cyclodextrin. Similarly, the hydrophobicity of the interior can be altered through substitution, though generally the hydrophobic nature of the interior allows accommodation of relatively hydrophobic guests within the cavity. Accommodation of one molecule within another is known as complexation and the resulting product is referred to as an inclusion complex. Examples of cyclodextrin derivatives include sulfobutylcyclodextrin, maltosylcyclodextrin, hydroxypropylcyclodextrin, and salts thereof.

Alternatively, the pharmaceutically appropriate form of antioxidant compound may be formulated so as to enhance the stability and bioavailability of the antioxidant compound. For example, enteric coatings may be applied to tablets to prevent the release of the antioxidant compound in the stomach either to reduce the risk of unpleasant side effects or to maintain the stability of the antioxidant compound which might otherwise be subject to degradation of expose to the gastric environment. Most polymers that are used for this purpose are polyacids that function by virtue or the fact that their solubility in aqueous medium is pH-dependent, and they require conditions with a pH higher then normally encountered in the stomach.

One preferable type of oral controlled release structure is enteric coating of a solid dosage form. Enteric coatings promote the compounds remaining physically incorporated in

the dosage form for a specified period when exposed to gastric juice. Yet the enteric coatings are designed to disintegrate in intestinal fluid for ready absorption. Delay of absorption is dependent on the rate of transfer through the gastrointestinal tract, and so the rate of gastric emptying is an important factor. For some administrations, a multiple-unit type dosage form, such as granules, may be superior to a single-unit type. Therefore, in one embodiment, antioxidant compounds of the invention may be contained in an enterically coated multiple-unit dosage form. In a more preferable embodiment, the antioxidant compound dosage form is prepared by producing particles having an antioxidant compound -enteric coating agent solid on an inert core material. These granules can result in prolonged absorption of the antioxidant compound with good bioavailability.

Typical enteric coating agents include, but are not limited to, hydroxypropylmethylcellulose phthalate, methacryclic acid-methacrylic acid ester copolymer, polyvinyl acetate-phthalate and cellulose acetate phthalate.

It will be appreciated that for an antioxidant compound of the present invention, as for any drug, activity *in vitro* is by no means the sole determinant of efficacy *in vivo*. The antioxidant activity of the antioxidant compounds of the present invention can be determined by methods such as those described herein using, for example, isolated mitochondria and isolated cells. Whilst it is true that, to be useful as a mitochondrially targeted antioxidant compound of the present invention, an antioxidant compounds must exhibit a suitably high antioxidant activity in such assays, to be efficacious *in vivo* the mitochondrially targeted antioxidant compounds must exhibit other desirable physicochemical properties, for example, suitably high bioavailability, or stability.

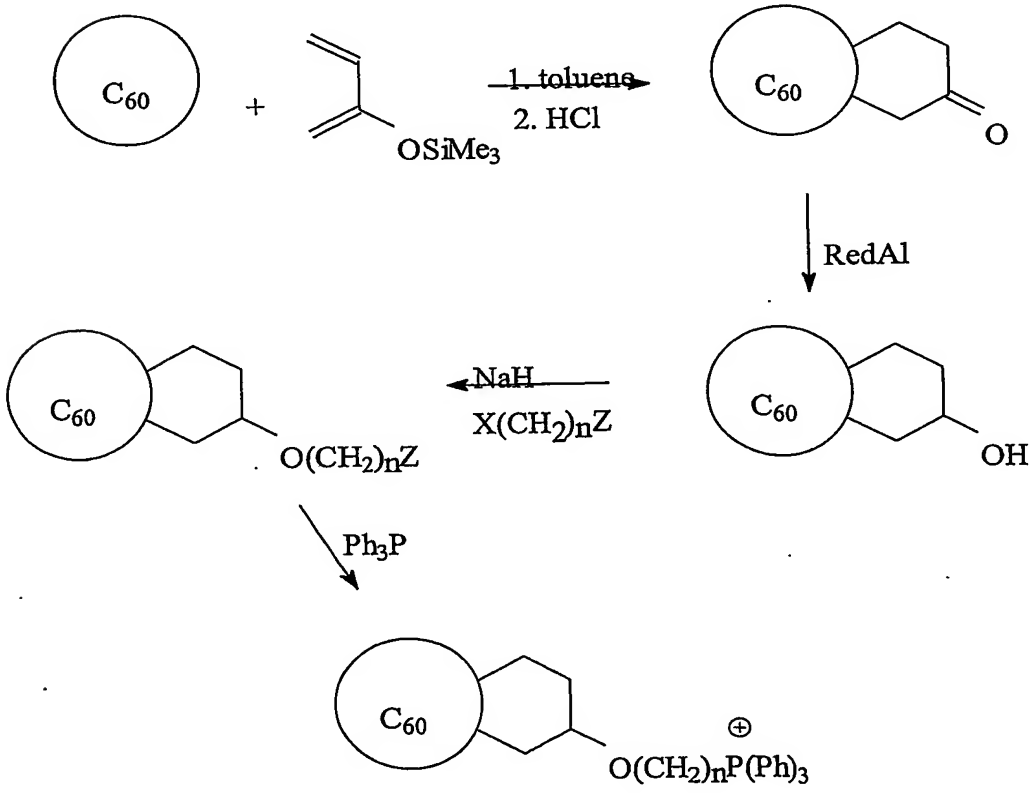
Examples of antioxidant compounds that show good antioxidant activity yet exhibit poor bioavailability with respect to the target compartment *in vivo* include Coenzyme Q (CoQ) and Idebenone. Both of these compounds must be administered at very high dose rates (for example, 0.5 – 1.2g) to obtain minimal clinical effects in human patients.

Examples of the mitochondrially targeted antioxidant compounds of the present invention exhibit good antioxidant activity and, at least in part by virtue of their low partition co-efficient, excellent bioavailability and thereby are efficacious *in vivo* at low dose rates. We believe the antioxidant compounds of the present invention to be equally effective at mitochondrial targeting, whilst exhibiting one or more of the additional benefits of being available as a crystalline or solid form, stable, or exhibiting low partition coefficients in turn leading, we believe (without wishing to be bound by any theory), to enhanced bioavailability.

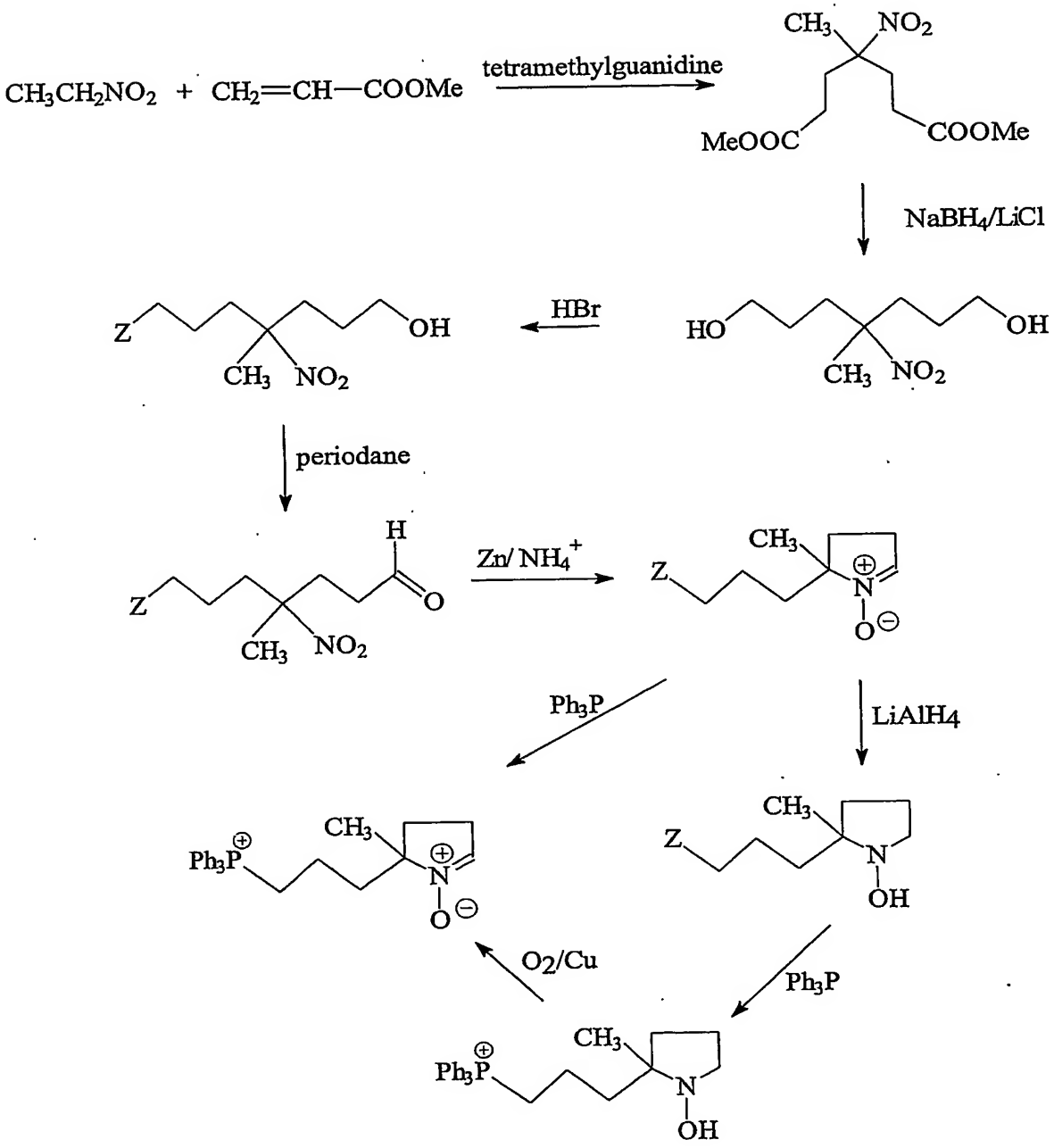
Such physical and chemical characteristics, we believe, again without wishing to be bound by any theory, confer upon the antioxidant compounds of the present invention preferred characteristics, thereby enabling their use in compositions, formulations and methods amongst other applications to which the antioxidant compounds of the prior art may be less suitable given their chemical and physical properties.

Set out below are synthetic schemes which may be used to prepare some other specific mitochondrially targeted antioxidant compounds of the present invention, namely (A) a mitochondrially targeted version of buckminsterfullerene; (B) a mitochondrially targeted spin trap compound; and (C) a further synthetic route for a mitochondrially targeted spin trap compound.

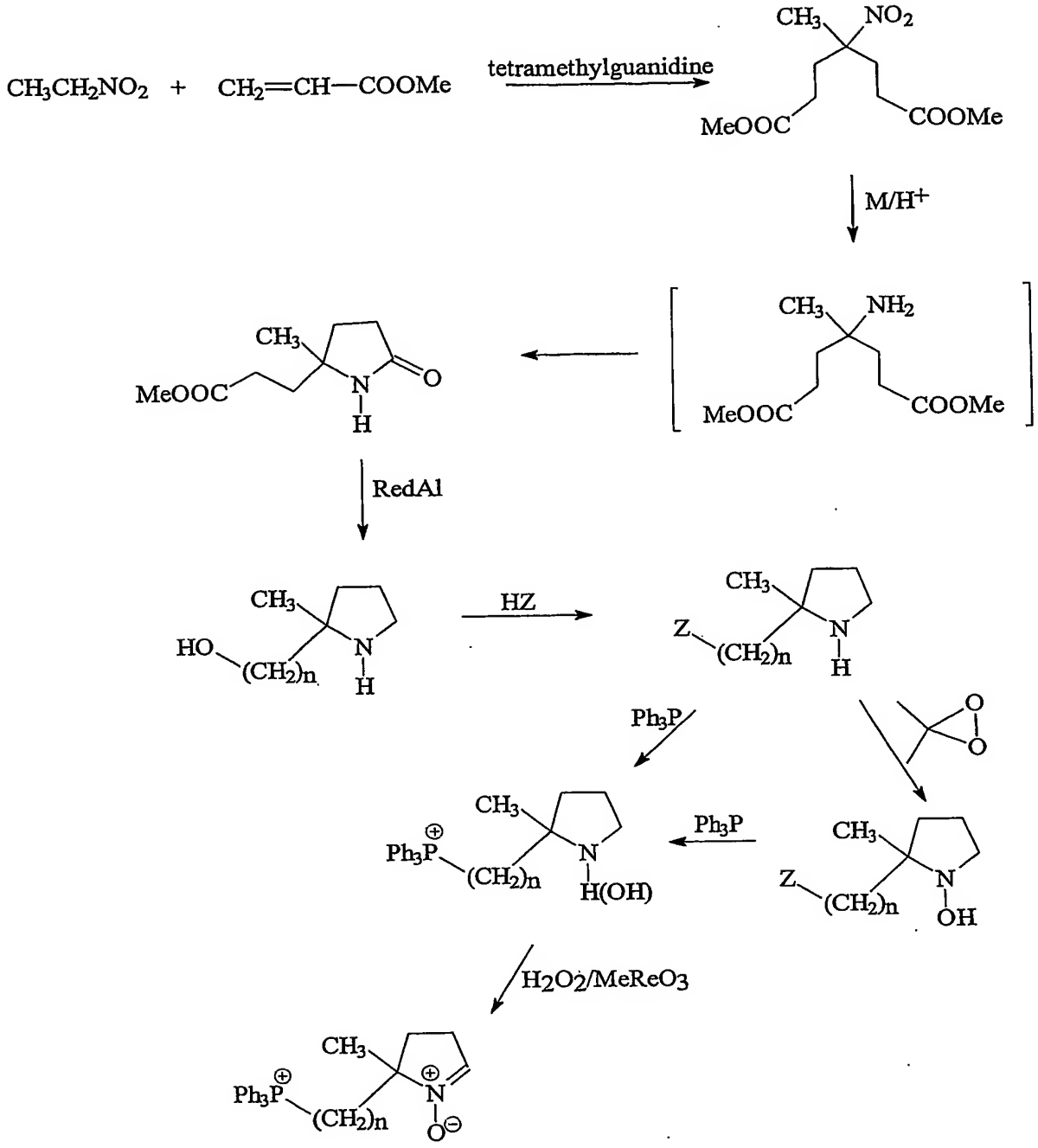
Buckminsterfullerene Synthesis



Spin Trap Synthesis I



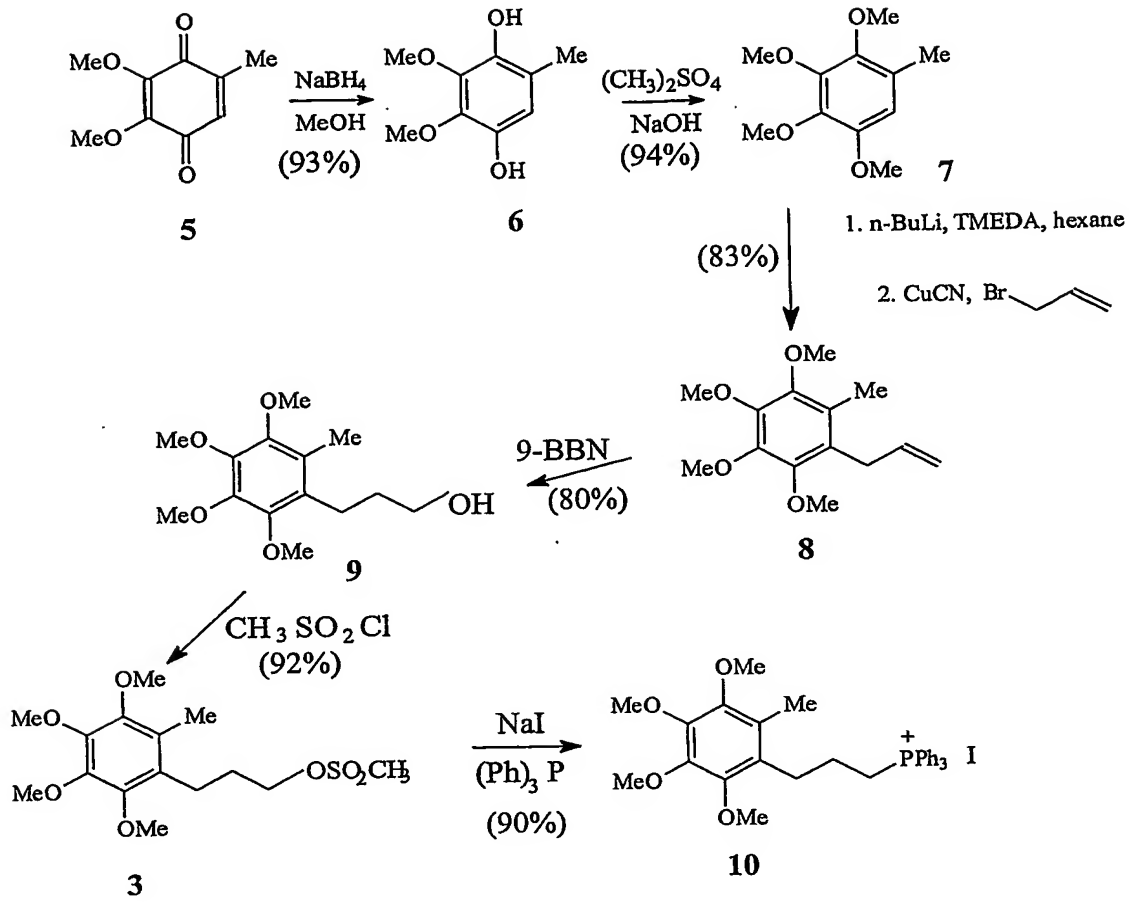
Spin Trap Synthesis II



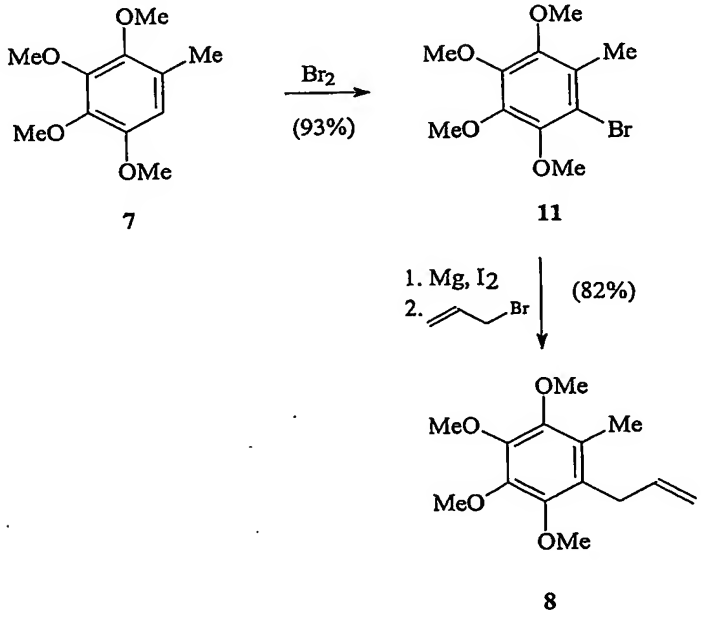
Set out below is the synthetic scheme which may be used to prepare the preferred mitochondrially targeted antioxidant compound of the present invention, namely compound **(2)**.

Preferably the antioxidant compound of the invention will exhibit good bioavailability. Bioavailability is at least in part dependent upon the partition coefficient of the antioxidant compound. Generally, those antioxidant compounds with a low partition coefficient (octanol:water) will be more readily bioavailable than those antioxidant compounds with a high partition coefficient (see table 1). Preferably the antioxidant compounds will have a partition coefficient (octanol:water) that lies in the range of about 0 to about 20, more preferably about 1 to about 10, more preferably a partition coefficient that lies in the range of about 2 to about 5.

An overall synthetic route to compound **(2)**is shown in Schemes 1 and 3 and is based on converting 4,5-dimethoxy-5-methylbenzoquinone (CoQ_0) **(5)** to 2,3,4,5-tetramethoxytoluene **(7)** then adding a three carbon side chain by metallation and reaction with allyl bromide. Compound (7) has been prepared by alternative routes based on *p*-cresol (Keinan, E.; Eren, D., 1987 *J Org Chem* , **52**, 3872). There are two possible metallation routes: lithiation of **7** followed by copper-catalysed allylation as shown in Scheme 1 or *via* the bromo derivative **(11)** followed by Grignard formation and allylation (Yoshioka, T., Nishi, T., Kanai, T., Aizawa, Y., Wada, K., Fujita, T., and Horikoshi, H. 1993 EP 549366 A1 19930630 CAN 119:225944) (Scheme 2). Hydroboration of **8** gives the alcohol **9** which is activated as a mesylate **(3)** and displaced with triphenylphosphine to give the aromatic phosphonium salt **10** iodide. The phosphonium salt formation is best carried out in the absence of solvent using sodium iodide to enhance the reaction.

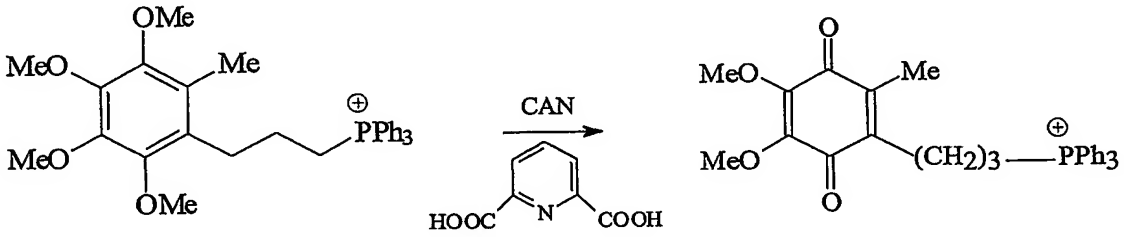


Scheme 1



Scheme 2

Conversion of the tetramethoxyaromatic ring in **10** into the required dimethoxyquinone (**2**) is achieved by using ceric ammonium nitrate (CAN) on the nitrate salt of **10** (Scheme 3). The product is obtained as a nitrate salt and anion exchange gives the target molecule mQ_3 OMs.



10 **2**

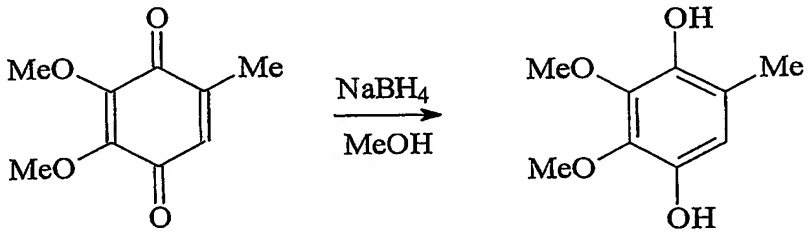
Scheme 3

The invention will now be described in more detail with reference to the following non-limiting experimental section.

EXAMPLE 1.

Synthesis of a mitochondrially-targeted quinone/ol derivative (Compound 2)

3,4-Dimethoxy-5-methyl-1,4-benzenediol **(6)** (CAS Reg 3066-90-8)



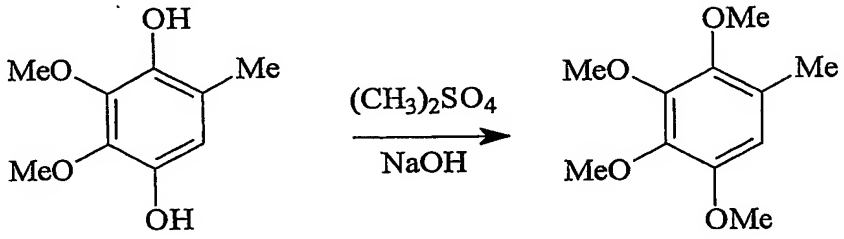
5 **6**

A solution of 2,3-dimethoxy-5-methyl-2,5-cyclohexadiene-1,4-dione (CoQ_0) (CAS Reg 605-94-7) (**5**) (14.62g, 80.25mmol) in a mixture of ether (220mL) and methanol (109mL) at room temperature was added dropwise to a stirred solution of $NaBH_4$ (15g, 396 mmol) in H_2O (440mL). The red color of the xidize (**5**) was changed to yellow on each addition. After stirring 15 mins at room temperature, the ether phase was separated and the aqueous phase extracted twice with ether (200mL). The combined ethereal extracts were washed with

saturated aqueous NaCl (300mL), dried ($MgSO_4$), filtered and evaporated *in vacuo* (20mm Hg) to give pure **6** as a yellow liquid (13.65g, 93%).

^{1}H NMR (299.9 MHz) δ 6.48 (1H, s, Ar-**H**), 3.91, 3.88 (6H, s, **Ome**), 2.17 (3H, s, **Me**).

1,2,3,4-Tetramethoxy-5-methyl-benzene (7) (CAS Reg 35896-58-3)

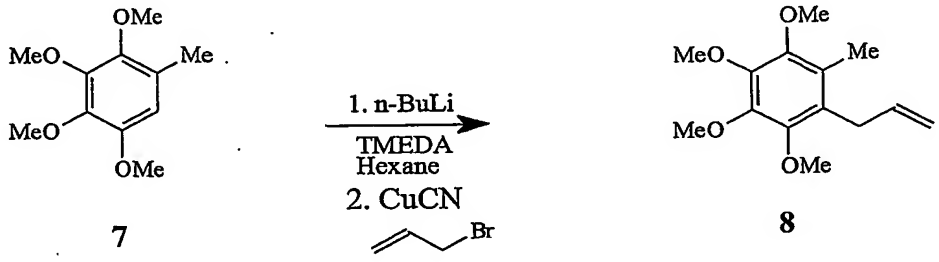


6 **7**

A solution of hydroquinone **(6)** (5.1g, 27.7 mmol) in ethanol (40 mL) was prepared at room temperature and an aqueous solution (40 mL) of NaOH (2.6g) was added in six portions simultaneously with dimethyl sulphate (6mL) with external room temperature water-cooling. After 45 min, 5% HCl (20mL) was added and the mixture was extracted with ethyl acetate (3x200mL). The organic layer was washed successively with water and brine, and dried ($MgSO_4$), filtered and then evaporated *in vacuo* to give a crude product **(7)** as a red oil (5.5g). Column chromatography of the crude product on silica gel (100g, packed in hexane) and elution with 15 % ether in hexane afforded pure 7 as a yellow oil (4.95g, 84 %).

^{1}H NMR (299.9 MHz) δ 6.44 (1H, s, Ar-**H**), 3.93, 3.87, 3.82, 3.79 (12H, s, **Ome**), 2.23 (3H, s, **Me**).

1,2,3,4-Tetramethoxy-5-methyl-6-(2-propenyl)benzene **(8)** (CAS Reg 71573-66-5) ***via Lithiation*** (Sakamoto, K., Miyoshi, H., Ohshima, M., Kuwabara, K., Kano, K., Akagi, T., Mogi, T., and Iwamura, H. 1998 *Biochemistry* **37** 15106).



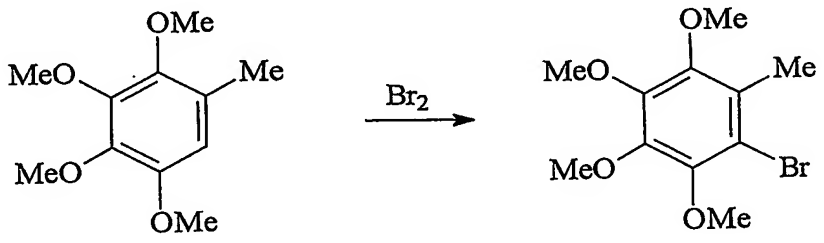
7 **8**

A solution of **7** (6.35 g, 29.9 mmol) in dry hexane (80 mL) and TMEDA (8.6 mL) was placed with a flame-dried stirrer bar in a flame-dried Schlenk tube under nitrogen. A hexanes solution of n-BuLi (1.6M, 26.2mL) was slowly added at room temperature and the mixture was cooled and stirred at 0^0 for 1hr. After being cooled to –78 ^{0}C, THF (250 mL) was added, and a small aliquot of the reaction mixture was removed, quenched with D_2O and examined by ^{1}H NMR to assure complete metallation. The yellow suspension was then completely transferred to a second flame-dried Schlenk tube containing CuCN (0.54 g) under nitrogen at –78 ^{0}C. The mixture was warmed to 0°C for 10 mins, then cooled to –78 ^{0}C and allyl bromide (3.62 mL) was added and the reaction was stirred overnight (19hrs) and allowed to warm to room temperature. The reaction was quenched with 10% aqueous NH_4Cl (75 mL), and extracted with ether (2 x 200 mL). The combined ethereal extracts were washed with H_2O (2 x 150 mL), 10% aqueous NH_4OH (200 mL) and saturated aqueous NaCl (200 mL). The organic solvents were dried over $MgSO_4$, filtered and the solvent removed by rotary evaporation *in vacuo* to give a crude product (7.25g). The ^{1}H NMR spectrum of this material indicated >93% product formation.

Column chromatography on silica gel and elution with 10:1 hexane:ethyl acetate gave pure **8** (6.05 g, 83.5 %).

^{1}H NMR (299.9 MHz) δ 5.84-5.98 (1H, m, -C**H**=C), 4.88-5.03 (2H, m, =C**H$_2$**), 3.78, 3.80, 3.90, 3.92 (12H, s, O**me**), 3.38 (2H, d, J= Hz, Ar-C**H$_2$**), 2.14 (3H, s, **Me**).

***1-Bromo-2,3,4,5-tetramethoxy-6-methylbenzen*e (11)** (CAS Reg 73875-27-1)



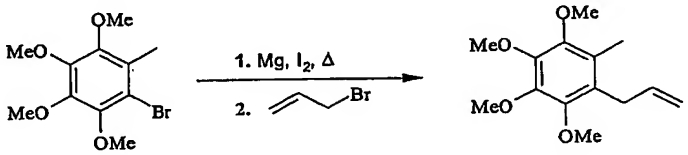
7 **11**

A solution of Br_2 (7.92 g, 4.9 mmol) in CH_2Cl_2 (10 mL) was slowly added to a solution of **7** (10g, 4.7 mmol) in CH_2Cl_2 (50 mL) at room temperature over 5 mins. The reaction was stirred for a further 5 mins, by which time t.l.c (20% ether:hexane) showed the reaction to be complete. The reaction was quenched with H_2O (50 mL), then washed with 5% aqueous NaOH (50 mL) and saturated NaCl (50 mL). The organic fraction was dried ($MgSO_4$), filtered

and the solvent removed *in vacuo* to give the crude product as an orange oil (13.79g). Chromatography on silica gel (180 g) and elution with 10% ether:hexane gave pure **11** (12.72 g, 93%) as a pale yellow oil.

^{1}H NMR (299.9 MHz) δ 3.91 (6H, s, **Ome**), 3.85 (3H, s, **Ome**), 3.79 (3H, s, **Ome**), 2.30 (3H, s, Ar-**Me**).

1,2,3,4-Tetramethoxy-5-methyl-6-(2-propenyl)benzene **(8)** (CAS Reg 71573-66-5) ***via Grignard***(Yoshioka, T., Nishi, T., Kanai, T., Aizawa, Y., Wada, K., Fujita, T., and Horikoshi, H. 1993 EP 549366 A1 19930630 CAN 119:225944)

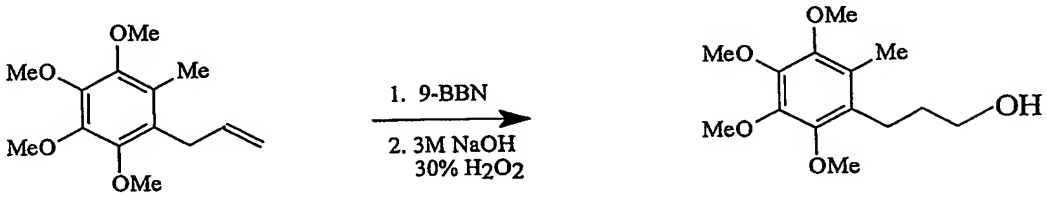


11 **8**

Compound **11** (15.26 g, 5.2 mmol) was dissolved in freshly distilled (Na,K) THF (70 mL) in a flame-dried Schlenk tube under argon. Magnesium (1.91 g, 7.8 mmol) was ground in a mortar and pestle and added to another flame-dried Schlenk tube under argon. The Mg was flame-dried under vacuum and, after cooling, a small amount (~20 mg) of Iodine and freshly distilled THF (10 mL) was added. The magnesium mixture was stirred for 1 min at 45 °C in which time the solution became turbid and then 10 mL of the THF solution of **11** was added under argon *via* a cannula. The resultant solution quickly lost its turbidity and became pale yellow. The remaining solution of **11** was added in 10 mL portions over 10 mins at 45 °C and then the solution was stirred for a further 20 mins at room temperature. A small aliquot of the reaction mixture was quenched at this time with D_2O and t.l.c (20% ether:hexane) showed all of **11** had been reacted. ^{1}H NMR indicated 85% deuterium incorporation in the product **7**. Allyl bromide (9 mL, 10.4 mmol) was then added by syringe and the reaction was stirred for 3 hrs at room temperature. The solvent was removed *in vacuo* and saturated aqueous NH_4Cl (100 mL) was added. The mixture was then extracted with ethyl acetate (4 × 100 mL) and the extract dried ($MgSO_4$) and filtered. The solvent was removed *in vacuo* to give the crude product as a pale orange oil. Purification by silica gel column chromatography (200g) eluting with 10:1 hexane:ethyl acetate gave **8** (10.30 g, 82 %) as a colourless oil together with a small impure fraction.

^{1}H NMR (299.9 MHz) δ 5.84-5.98 (1H, m, C**H**=C), 4.88-5.03 (2H, m, C**H$_2$**=C), 3.78, 3.80, 3.90, 3.92, (12H, s, **Ome**), 3.38 (2H, d, Ar-C**H$_2$**-C), 2.14 (3H, s, Ar-**Me**).

3-(2,3,4,5-Tetramethoxy-6-methyl-phenyl)-propan-1-ol (9)

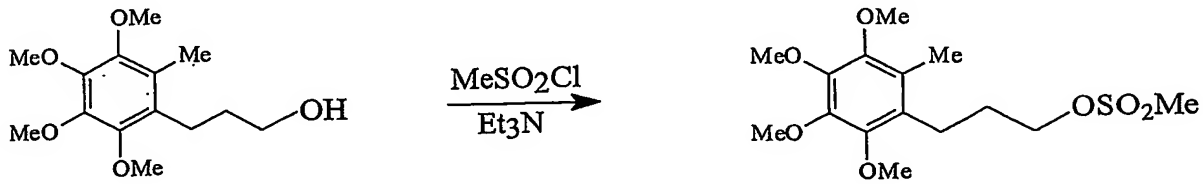


8 **9**

A solution of **8** (8.0 g, 33.05 mmol) in THF (45mL) was added dropwise over 20 mins under argon to a stirred suspension of 9-borabicyclo[3,3,1]nonane (9-BBN) in THF (79mL, 39.67mmol, 0.5M) at 25°. The resulting solution was stirred overnight at room temperature and for further 2hrs at 65° under argon. The mixture was then cooled to 0° and 3M NaOH (53 mL) was then added dropwise followed by 30% aqueous H_2O_2 (53 mL). After 30 mins stirring at room temperature, the water phase was saturated with NaCl and extracted 3 times with THF. The combined organic fractions were washed with saturated aqueous NaCl, dried (Na_2SO_4), filtered and evaporated to give an oily residue (11.5g) which was purified by column chromatography on silica gel (200 g, packed with ether/hexane 1:9). Elution with ether/hexane 1:4 gave pure **9** as viscous, colourless oil (6.85 g, 80%).

^{1}H NMR (299.9 MHz) δ 3.91, 3.90, 3.84, 3.79 (12H, s, **Ome**), 3.56 (2H, t, J=7.0Hz, -C**H$_2$**-OH), 2.72 (2H, t, J=7.0 Hz, Ar-C**H$_2$**), 2.17 (3H, s, Ar-C**H$_3$**), 1.74 (2H, quintet, J=7.0 Hz, -C**H$_2$**-).

1-Methanesulfonyloxy-3-(2,3,4,5-tetramethoxy-6-methyl-phenyl)-propane (3)

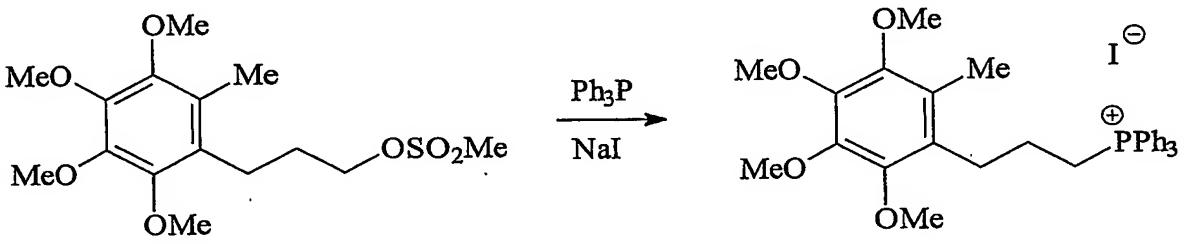


9 **3**

A solution of **9** (3.88 g, 15 mmol) and triethylamine (3.0 g, 30 mmol, 4.2mL) in CH_2Cl_2 (50 mL) was stirred at room temperature for 10 mins. Methane sulfonyl chloride (1.8 g, 1.20mL, 15.75 mmol) in CH_2Cl_2 (50 mL) was added dropwise over 20 mins and the reaction mixture stirred at room temperature for 1 hr. The mixture was then diluted with CH_2Cl_2 (50 mL) and the organic layer was washed with H_2O (5 x 100 mL), 10% aqueous $NaHCO_3$ (100 mL), dried ($MgSO_4$), filtered and the solvent removed *in vacuo* by rotary evaporation to afford pure **3** as a liquid (4.8 g, 95 %).

^1H NMR (299.9 MHz) δ 4.27 (2H, t, J = 7.0 Hz, -CH_2-O-SO_2-Me), 3.91, 3.89, 3.82, 3.78 (12H, s, **Ome**), 3.03 (3H, s, -O-SO_2-**Me**), 2.70 (2H, t, J = 7.0 Hz, Ar-CH_2-), 2.17 (3H, s, **Me**), 1.9 (2H, m, -CH_2-).

[3-(2,3,4,5-Tetramethoxy-6-methyl-phenyl)-propyl]triphenylphosphonium iodide **(10)**

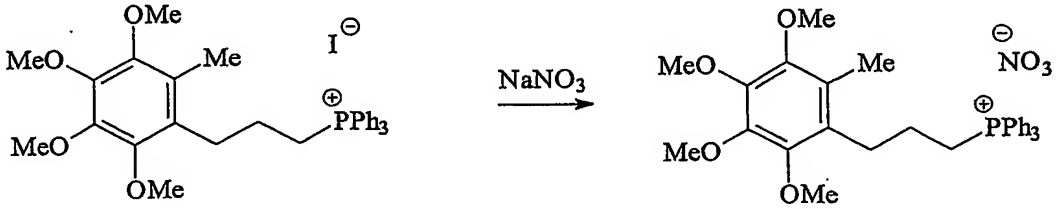


3 **10**

The methanesulfonate **3** (3.30 g, 9.8 mmol) was mixed with a freshly ground mixture of triphenylphosphine (4.08 g, 15.6 mmol) and NaI (7.78 g, 51.9 mmol) in a KIMAX tube and sealed under argon. The mixture was then held at 70-74 °C with magnetic stirring for 3 hrs during which time the mixture changed from a molten thick liquid into a glassy solid. The tube was cooled to room temperature and the residue stirred with CH_2Cl_2 (30 mL). The suspension was then filtered and the filtrate evaporated *in vacuo*. The residue was dissolved in a minimum amount of CH_2Cl_2 and triturated with excess ether (250 mL) to precipitate the white solid. The solid was filtered and washed with ether, dried *in vacuo* to give pure **10** (5.69 g, 90%).

^1H NMR (299.9 MHz) δ 7.82-7.65 (15H, m, Ar-**H**), 3.88, 3.86, 3.74, 3.73 (12H, s, **Ome**), 3.76-3.88 (2H, m, CH_2-P^+), 2.98 (2H, t, J=7.0 Hz, CH_2-Ar), 2.13 (3H, s, Ar-CH_3), 1.92-1.78 (2H, m, -CH_2-). ^{31}P NMR (121.4 MHz) δ 25.32 (-CH_2-P^+Ph_3).

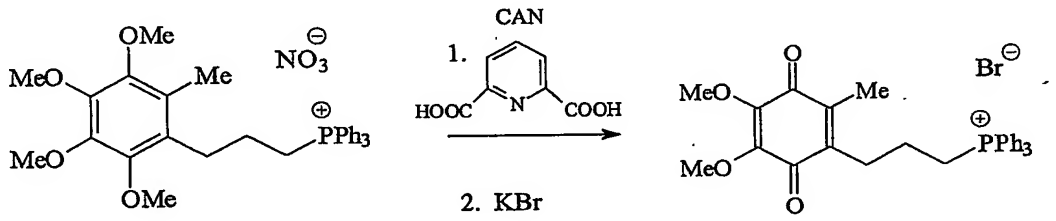
***[3-(2,3,4,5-Tetramethoxy-6-methyl-phenyl)-propyl]triphenylphosphonium nitrate* (10)**



10 **10**

A solution of the iodide form of **10** (4.963 g, 7.8 mmol) in CH_2Cl_2 (80mL) was shaken with 10% aqueous $NaNO_3$ (50 mL) in a seporatory funnel for 5 mins. The organic layer was separated, dried (Na_2SO_4), filtered and evaporated *in vacuo* to give the crude nitrate form of **10** (4.5 g, 100%).

***[3-(4,5-Dimethoxy-2-methyl-3,6-dioxo-1,4-cyclohexadien-1-yl)propyl]triphenylphosphonium bromide* (2)**

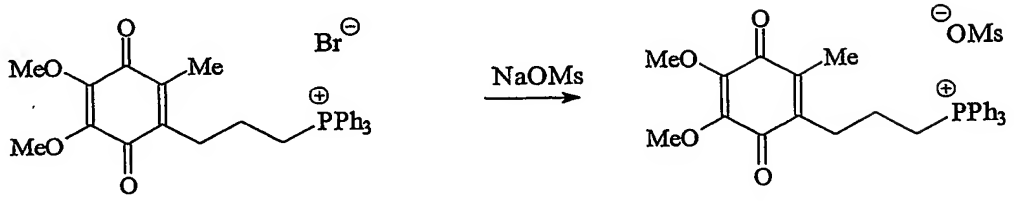


10 **2**

The nitrate form of **10** (4.5 g, 7.8 mmol) was dissolved in a mixture of CH_3CN and H_2O (7:3, 38 mL) and stirred at 0°C in an ice bath. Pyridine-2,6-dicarboxylic acid (6.4 g, 39 mmol) was then added followed by drop wise addition of a solution of ceric ammonium nitrate (21.0 g, 39 mmol) in CH_3CN/H_2O (1:1, 77 mL) over 5 mins. The reaction mixture was stirred at 0°C for 20 mins and then at room temperature for a further 10 mins. The reaction mixture was then poured into H_2O (200 mL) and extracted with CH_2Cl_2 (200 mL), dried (Na_2SO_4), filtered and evaporated *in vacuo* to give a crude **2** nitrate. The total product was dissolved in CH_2Cl_2 (100 mL) and shaken for 10 mins with 20% aqueous KBr (50 mL). The organic layer was separated, dried and evaporated *in vacuo* to give NMR pure **2** bromide (4.1 g, 93.6%).

^1H NMR (299.9 MHz) δ 7.90-7.65 (15H, m, Ar-**H**), 4.15-4.05 (2H, m, CH_2-P^+), 3.96, 3.95, (6H, s, Ome), 2.93 (2H, t, J=7.0 Hz, CH_2-Ar), 2.15 (3H, s, Ar-CH_3), 1.85-1.70 (2H, m, -CH_2-). ^{31}P NMR (121.4 MHz) δ 25.29 (-CH_2-P^+Ph_3).

[3-(4,5-Dimethoxy-2-methyl-3,6-dioxo-1,4-cyclohexadien-1-yl)propyl]triphenylphosphonium methane sulfonate (2)



2 2

A solution of **2** bromide (3.65g, 6.5 mmol) in CH_2Cl_2 (75 mL) was shaken with an aqueous solution of sodium methane sulphonate (100 mL) in a seporatory funnel for 5 mins. The CH_2Cl_2 layer was separated, dried (Na_2SO_4), filtered and evaporated *in vacuo* to give the methane sulfonate salt of **2** (3.7 g, 98%).

^{1}H NMR (299.9 MHz) δ 7.88-7.60 (15H, m, Ar-**H**), 3.93, 3.92, (6H, s, **Ome**), 3.90-3.78 (2H, m, CH_2-P^+), 2.85 (2H, t, J=7.0 Hz, CH_2-Ar), 2.70 (3H, s, OSO_2CH_3), 2.09 (3H, s, Ar-CH_3), 1.82-1.68 (2H, m, -CH_2-). ^{31}P NMR (121.4 MHz) δ 25.26 (-CH_2-P^+Ph_3).

EXAMPLE 2. Properties of mitochondrially-targeted antioxidant compounds of the present invention

The present invention recognises that, in order to be suitable in a wide variety of applications, for example the formulation of dosage forms such as tablets, there is advantage in being able to form a crystalline or solid form of the mitochondrially-targeted antioxidant compound. Similarly, it is believed, without wishing to be bound by any theory, that mitochondrially-targeted antioxidant compounds with low partition coefficients (octanol:water)may exhibit enhanced bioavailability with respect to those compounds having higher partition coefficients, such that a low partition coefficient is desirable for certain applications.

The partition coefficients for a variety of antioxidant compounds are shown in Table 1. Of particular note is the low partition coefficient of compounds with small numbers of carbon atoms bridging the antioxidant compoment and the phosphonium. For example, a compound within the present invention, herein referred to as Mitoquinone-C5, which has a 5

carbon bridge has a partition coefficient approximately 50-fold lower than that observed for the related compound, Mitoquinone-C10 (Table 1).

TABLE I. Partition Coefficients of Coenzyme Q, Targeted Derivatives and Related Compounds

CompoundPartition coefficient	
Methyltriphenylphosphonium (TPMP)	[a] 0.35 ± 0.02
MitoVit E	[b] 7.4 ± 1.6
4-Bromobutyltriphenylphosphonium	[b] 3.83 ± 0.22
4-Iodobutyltriphenylphosphonium	[c] 4.0 ± 0.4
Mitoquinone-C10	[a] 160 ± 9
Mitoquinone-C5	[c] 2.8 ± 0.3
α-Tocopherol	[b] 27.4 ± 1.9
Bromodecylubiquinone	[d] 310 ± 60
Idebenone	[d] 3.1×10^3
Decylubiquinone	[d] 3.1×10^5
Coenzyme Q_0	[d] 1.33
Coenzyme Q_1	[d] 409
Coenzyme Q_2	[d] 4.44×10^4
Ubiquinone (Coenzyme Q_{10})	[d] 1.82×10^{20}
Ubiquinol	[d] 4.53×10^{20}
Decylubiquinol	[d] 7.91×10^5
Idebenol	[d] 7.82×10^3

Data[a-c] are octan-1-ol/phosphate buffered saline partition coefficients determined at 25°C or 37°C, or octanol/water partition coefficients[d] calculated using Advanced Chemistry Development (ACD) Software Solaris V4.67 as described in Jauslin, M. L., Wirth, T., Meier, T., and Schoumacher, F., 2002, *Hum Mol Genet* **11**, 3055.

[a] Kelso, G.F., Porteous, C.M., Coulter, C. V., Hughes, G., Porteus, W. K., Ledgerwood, E. C., Smith, R. A. J., and Murphy, M. P., 2001, *J Biol Chem* **276**, 4588.

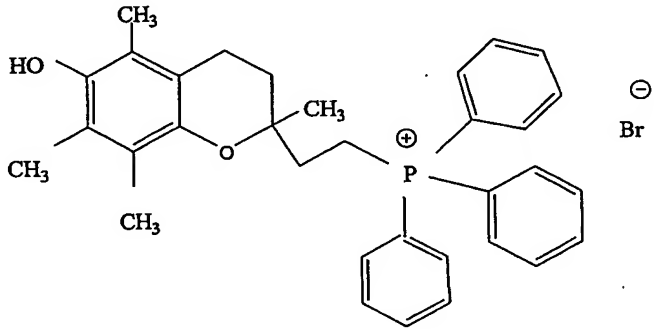
[b] Smith, R. A. J., Porteous, C. M., Coulter, C. V., and Murphy, M. P. 1999 *Eur J Biochem* **263**, 709.

[c] Smith, R.A.J., Porteous, C.M., Gane, A.M., and Murphy, M.P. 2003 *Proc Nat Acad Sci* **100**, 9, 5407.

EXAMPLE 3.

Mitochondrial uptake of mitochondrially-targeted compounds

To demonstrate that mitochondrial targeting is effective, the exemplary vitamin E compound, compound (4), was tested in relation to both isolated mitochondria and isolated cells.



(4)

To do this a [^{3}H]-version of compound (4) was synthesized using [^{3}H]-triphenylphosphine and the mitochondrial accumulation of compound (4) quantitated by scintillation counting (Figure1) (Burns *et al.*, 1995, *Arch Biochem Biophys* **332**, 60. Burns and Murphy, 1997, *Arch Biochem Biophys* **339**, 33). To do this rat liver mitochondria were incubated under conditions known to generate a mitochondrial membrane potential of about 180 mV (Burns *et al.*, 1995, *Arch Biochem Biophys* **332**, 60. Burns and Murphy, 1997, *Arch Biochem Biophys* **339**, 33). Under these conditions compound 4 was rapidly (< 10 s) taken up into mitochondria with an accumulation ratio of about 6,000. This accumulation of compound 4 into mitochondria was blocked by addition of the uncoupler FCCP (carbonyl

cyanide-p-trifluoromethoxyphenylhydrazone) which prevents mitochondria establishing a membrane potential (Figures 1 and 2) (Burns *et al.*, 1995, *Arch Biochem Biophys* **332**, 60.). Therefore compound **4** is rapidly and selectively accumulated into mitochondria driven by the mitochondrial membrane potential and this accumulation results in a concentration of the compound within mitochondria several thousand fold higher than in the external medium. This accumulation is rapidly (<10 s) reversed by addition of the uncoupler FCCP to dissipate the mitochondrial membrane potential after accumulation of compound **4** within the mitochondria. Therefore the mitochondrial specific accumulation is solely due to the mitochondrial membrane potential and is not due to specific binding or covalent interaction.

The mitochondrial specific accumulation of compound **4** also occurs in intact cells. This was measured as described by Burns and Murphy, 1997, *Arch Biochem Biophys* **339**, 33 and the accumulation was prevented by dissipating both the mitochondrial and plasma membrane potentials. In addition, compound **4** was not accumulated by cells containing defective mitochondria, which consequently do not have a mitochondrial membrane potential. Therefore the accumulation of compound **4** into cells is driven by the mitochondrial membrane potential.

The accumulation ratio was similar across a range of concentrations of compound **4** and the amount of compound **4** taken inside the mitochondria corresponds to an intramitochondrial concentration of 4-8 mM (Fig 2). This uptake was entirely due to the membrane potential and paralleled that of the simple triphenylphosphonium cation TPMP over a range of membrane potentials (Fig 3). From comparison of the uptake of TPMP and compound **4** at the same membrane potential we infer that within mitochondria about 84% of compound **4** is membrane-bound Further details of the experimental procedures and results are given below.

Figure 1 shows the uptake of 10 μM [^{3}H] compound **4** by energised rat liver mitochondria (continuous line and filled symbols). The dotted line and open symbols show the effect of addition of 333 nM FCCP at 3 min. Incubation with FCCP from the start of the incubation led to the same uptake as for adding FCCP at 3 min (data not shown). Liver mitochondria were prepared from female Wistar rats by homogenisation followed by differential centrifugation in medium containing 250 mM sucrose, 10 mM Tris-HCL (pH 7.4) and 1mM EGTA and the protein concentration determined by the biuret assay using BSA as a standard. To measure [^{3}H] compound **4** uptake mitochondria (2 mg protein/ml) were suspended at 25°C in 0.5 – 1 ml 110 mM KCl, 40 mM Hepes-KOH, pH 7.2, 0.1 mM EDTA

supplemented with nigericin (1 μg/ml), 10 mM succinate, rotenone 1.33 μg/ml and 60 nCi/ml [^{3}H] compound V and 10 μM compound 4. After the incubation mitochondria were pelleted by centrifugation and the [^{3}H] compound 4 in the supernatant and pellet quantitated by scintillation counting.

Figure 2 shows the mitochondrial accumulation ratios [(compound 4/mg protein)/(compound 4/μl)] obtained following 3 min incubation of energised rat liver mitochondria with different concentrations of compound 4 (filed bars) and the effect of 333 nM FCCP on these (open bars). The dotted line and open circles show compound 4 uptake by mitochondria, corrected for FCCP-insensitive binding. To measure [^{3}H] compound 4 accumulation ratio mitochondria (2 mg protein/ml) were suspended at 25°C in 0.5 – 1 ml 110 mM KCl, 40 mM Hepes-KOH, pH 7.2, 0.1 mM EDTA supplemented with nigericin (1 μg/ml), 10mM succinate, rotenone 1.33 μg/ml and 6 – 60 nCi/ml [^{3}H] compound 4 and 1-50 μM compound 4. After the incubation mitochondria were pelleted by centrifugation and the [^{3}H] compound 4 in the supernatant and pellet quantitated by scintillation counting.

Figure 3 shows a comparison of compound 4 uptake with that of TPMP at a range of mitochondrial membrane potentials. Energised rat liver mitochondria were incubated for 3 min with 10 μM compound 4 and 1 μM TPMP and different membrane potentials established with 0-8 mM malonate or 333 nM FCCP. The accumulation ratios of parallel incubations with either 60 nCi/ml [^{3}H] compound 4 or 50 nCi/ml [^{3}H] TPMP were determined, and the accumulation ratio for compound 4 is plotted relative to that of TPMP at the same membrane potential (slope = 2.472, y intercept = 319, r = 0.97). Mitochondria (2 mg protein/ml) were suspended at 25°C in 0.5-1 ml 110 mM KCl, 40 mM Hepes-KOH, pH 7.2, 0.1 mM EDTA supplemented with nigericin (1μg/ml), 10 mM succinate, rotenone 1.33 μg/ml.

EXAMPLE 4.

Antioxidant efficacy of compound 4

The compounds of the invention are also highly effective against oxidative stress. To demonstrate this, exemplary compound 4 was further tested using rat brain homogenates. The rat brain homogenates were incubated with or without various concentrations of the test compounds (compound 4; native Vitamin E (α-tocopherol), bromobutyl triphenylphosphonium bromide, Trolox (a water soluble form of Vitamin E) and 2-(2-bromoethyl)-3,4-dihydro-2,5,7,8-tetramethyl-2H-1-benzopyran-6-ol, the precursor of compound 4 ("Brom Vit E")) and the oxidative damage occurring over the incubation was

quantitated using the TBARS assay (Stocks *et al.*, 1974, *Clin Sci Mol Med* 47,215). From this the concentration of compound required to inhibit oxidative damage by 50% was determined. In this system 210 nM compound 4 inhibited oxidative stress by 50% while the corresponding value for native Vitamin E was 36 nM. The value for bromobutyltriphenylphosphonium bromide, which contains the triphenylphosphonium moiety but not the antioxidant Vitamin E moiety was 47 μM. These data show that compound 4 is an extremely effective antioxidant, within an order of magnitude as effective as Vitamin E. Comparison with bromobutyltriphenylphosphonium bromide shows that the antioxidant capability is due to the Vitamin E function and not to the phosphonium salt. Further details of the experimental procedures and results are set out below.

The IC_{50} values for inhibition of lipid peroxidation were determined in rat brain homogenates, and are means ± SEM or range of determinations on 2-3 brain preparations. Octan-1-ol/PBS partition coefficients are means ± SEM for three independent determinations. N.D. not determined. Partition coefficients were determined by mixing 200 μM of the compound in 2 ml water-saturated octanol-1-ol with 2 ml octanol-saturated-PBS at room temperature for 1 h, then the two layers were separated by brief centrifugation and their concentrations determined spectrophotometrically from standard curves prepared in PBS or octanol. To measure antioxidant efficacy four rat brains were homogenised in 15 ml 40 mM potassium phosphate (pH 7.4), 140 mM NaCl at 4°C, particulate matter was pelleted (1,000 x g at 4°C for 15 min) and washed once and the combined supernatants stored frozen. Aliquots were rapidly thawed and 5 mg protein suspended in 800 μl PBS containing antioxidant or ethanol carrier and incubated at 37°C for 30 min. Thiobarbituric acid reactive species (TBARS) were quantitated at 532 nm by adding 200 μl conc. $HclO_4$ and 200 μl 1% thiobarbituric acid to the incubation, heating at 100°C for 15 min and then cooling and clarification by centrifugation (10,000 x g for 2 min). The results are shown in Table 2 below.

Table 2. Partition coefficients and antioxidant efficacy of compound 4 and related compounds

Compound	IC_{50} for inhibition of lipid peroxidation (nM)	Octanol:PBS partition coefficient

Compound 4	210 ± 58	7.37 ± 1.56
Bromo Vit E	45 ± 26	33.1 ± 4.4
α-Tocopherol	36 ± 22	27.4 ± 1.0
Trolox	18500 ± 5900	N.D.
BrBTP	47000 ± 13000	3.83 ± 0.22

When mitochondria were exposed to oxidative stress compound **4** protected them against oxidative damage, measured by lipid peroxidation and protein carbonyl formation (Figure 4). This antioxidant protection was prevented by incubating mitochondria with the uncoupler FCCP to prevent uptake of compound **4**, and lipophilic cations alone did not protect mitochondria (Figure 5). Most importantly, the uptake of compound **4** protected mitochondrial function, measured by the ability to generate a membrane potential, far more effectively than Vitamin E itself (Figure 6). This shows that the accumulation of compound **4** into mitochondria selectively protects their function from oxidative damage. In addition, we showed that compound **4** is not damaging to mitochondria at the concentrations that afford protection (Figure 7).

The next step was to determine whether compound **4** was accumulated by intact cells. Compound **4** was rapidly accumulated by intact 143B cells, and the amount accumulated was greater than that by ρ° cells derived from 143B cells. This is important because the ρ° cells lack mitochondrial DNA and consequently have far lower mitochondrial membrane potential than the 143B cells, but are identical in every other way, including plasma membrane potential, cell volume and protein content (Figure 8); this suggests that most of the compound **4** within cells is mitochondrial. A proportion of this uptake of compound **4** into cells was inhibited by blocking the plasma and mitochondrial membrane potentials (Figure9). This energisation-sensitive uptake corresponds to an intra mitochondrial concentration of compound **4** of about 2- 4 mM, which is sufficient to protect mitochondria from oxidative damage. These concentrations of compound **4** are not toxic to cells (Figure10).

Further details of the experimental procedures and results are discussed below.

Figure 4 shows the protection of mitochondria against oxidative damage by compound **4**. Mitochondria were exposed to oxidative stress by incubation with

iron/ascorbate and the effect of compound 4 on oxidative damage assessed by measuring TBARS (filled bars) and protein carbonyls (open bars). Rat liver mitochondria (10 mg protein) were incubated at 25°C in a shaking water bath in 2 ml medium containing 100 mM KCl, 10 mM Tris, pH 7.7, supplemented with rotenone (1.33 µg/ml), 10 mM succinate, 500 µM ascorbate and other additions. After preincubation for 5 min, 100 µM $FeSO_4$ was added and 45-55 min later duplicate samples were removed and assayed for TBARS or protein carbonyls.

Figure 5 shows a comparison of compound 4 with vitamin E and the effect of uncoupler and other lipophilic cations. Energised rat liver mitochondria were exposed to tert-butylhydroperoxide and the effect of compound 4 (filled bars), α-tocopherol (open bars), compound 4 + 333 nM FCCP (stippled bars) or the simple lipophilic cation bromobutyl triphenylphosphonium (cross hatched bars) on TBARS formation determined. Rat liver mitochondria (4 mg protein) were incubated in 2 ml medium containing 120 mM KCl, 10 mM Hepes-HCl pH 7.2, 1 mM EGTA at 37°C in a shaking water bath for 5 min with various additions, then tert butyl hydroperoxide (5 mM) was added, and the mitochondria incubated for a further 45 min and then TBARS determined.

Figure 6 shows how compound 4 protects mitochondrial function from oxidative damage. Energised rat liver mitochondria were incubated with iron/ascorbate with no additions (stippled bars), 5 µM compound 4 (filled bars), 5 µM α-tocopherol (open bars) or 5 µM TPMP (cross hatched bars), and then isolated and the membrane potential generated by respiratory substrates measured relative to control incubations in the absence of iron/ascorbate. Rat liver mitochondria were incubated at 25°C in a shaking water bath in 2 ml medium containing 100 mM KCl, 10 mM Tris, pH 7.7, supplemented with rotenone (1.33 µg/ml), 10 mM succinate, 500 µM ascorbate and other additions. After preincubation for 5 min, 100 µM $FeSO_4$ was added and after 30 min the incubation was diluted with 6 ml ice-cold STE 250 mM sucrose, 10 mM Tris-HCL (pH 7.4) and 1 mM EGTA, pelleted by centrifugation (5 min at 5,000 x g) and the pellet resuspended in 200 µl STE and 20 µl (= 1 mg protein) suspended in 1 ml 110 mM KCl, 40 mM HEPES, 0.1 M EDTA pH 7.2 containing 1 µM TPMP and 50 nCi/ml [3H] TPMP either 10 mM glutamate and malate, 10 mM succinate and rotenone, or 5 mM ascorbate/100 µM TMPD with rotenone and myxothiazol (2 µg/ml), incubated at 25°C for 3 min then pelleted and the membrane potential determined as above and compared with an incubation that had not been exposed to oxidative stress.

Figure 7 shows the effect of compound 4 on the membrane potential (filled bars) and respiration rate of coupled (open bars), phosphorylating (stippled bars) and uncoupled mitochondria (cross hatched bars), as a percentage of values in the absence of compound V. The effect of various concentrations of compound 4 on the membrane potential of isolated mitochondria was determined from the distribution of [^{3}H] TPMP by incubating rat liver mitochondria (2 mg protein/ml) in 0.5 ml medium as above containing 1 μM TPMP and 50 nCi/ml [^{3}H] TPMP at 25°C for 3 min. After the incubation mitochondria were pelleted by centrifugation and the [^{3}H] TPMP in the supernatant and pellet quantitated by scintillation counting and the membrane potential calculated assuming a volume of 0.5 μl/mg proteins and that 60% of intramitochondrial TPMP is membrane bound. To measure the effect of compound 4 on coupled, phosphorylating and uncoupled respiration rates, mitochondria (2 mg protein/ml) were suspended in 120 mM KCl, 10 mM Hepes-HCl pH 7.2, 1 mM EGTA, 10 mM K Pi in a 3 ml Clark oxygen electrode then respiratory substrate, ADP (200μM) and FCCP (333 nM) were added sequentially to the electrode and respiration rates measured.

Figure 8 shows the uptake of compound 4 by cells. Here 10^6 143B cells (closed symbols) or ρ° cells (open symbols) were incubated with 1 μM [3H] compound 4 and the compound 4 accumulation ratio determined. Human osteosarcoma 143B cells and a derived ρ° cell line lacking mitochondrial DNA were cultured in DMEM/10 % FCS (foetal calf serum) supplemented with uridine and pyruvate under an atmosphere of 5% CO_2/95% air at 37°C, grown to confluence and harvested for experiments by treatment with trypsin. To measure [^{3}H] compound 4 accumulation cells (10^6) were incubated in 1 ml HEPES-buffered DMEM. At the end of the incubation, cells were pelleted by centrifugation, the cell pellet and the supernatant prepared for scintillation counting and the accumulation ratio [compound 4/mg protein)/(compound 4/μl)] calculated.

Figure 9 shows the amount of compound 4 taken up by 10^6 143B cells over 1 h incubation, corrected for inhibitor-insensitive binding. Human osteosarcoma 143B cells were incubated in 1 ml HEPES-buffered DMEM with 1-50 μM compound 4 supplemented with 6-60 nCi/ml [^{3}H] compound 4. To determine the energistration-dependent uptake, parallel incubations with 12.5 μM oligomycin, 20 μM FCCP, 10 μM myxathiazol, 100 nM valinomycin and 1mM ouabain were carried out. At the end of the incubation, cells were pelleted by centrifugation and prepared for scintillation counting and the energisation-sensitive uptake determined.

Figure 10 shows the effect of compound **4** on cell viability. Here, confluent 143B cells in 24 well tissue culture dishes were incubated with various concentrations of compound **4** for 24 h and cell viability measured by lactate dehydrogenase release.

Figure. 11 shows the UV-absorption spectrum of [10-(6'-ubiquinonyl) decyltriphenylphosphonium bromide] (herein referred to as "the quinone form of compound **1**" or "mitoquinone") and of the reduced form of the compound [10-(6'-ubiquinonyl)decyltriphenylphosphonium bromide] (herein referred to as "the quinol form of compound **1**" or "mitoquinol").

Figures 12A to 12D show reactions of [10-(6'-ubiquinonyl) decyltriphenylphosphonium bromide (**1**)] ("mitoquinone") and the reduced form of the compound ("mitoquinol") with mitochondrial membranes. Beef heart mitochondrial membranes (20 mg/ml) were suspended in 50 mM sodium phosphate, pH 7.2 at 20°C. In panel A rotenone and antimycin were present and for the t=0 scan, then succinate (5 mM) was added and scans repeated at 5 minute intervals as indicated. In panel B A_{275} was monitored in the presence of rotenone and antimycin and then mitoquinone (50 mM) was added, followed by succinate (5 mM) and malonate (20 mM) where indicated. In Panel C rotenone, ferricytochrome c (50 mM) and malonate (20 mM) were present, A_{275} was monitored and mitoquinol (50 mM) and myxathiazol (10 mM) were added where indicated. In panel D, A 550 was monitored and the experiment in Panel C was repeated in the presence of KCN. Addition of myxathiazol inhibited this rate by about 60-70%. There was no reaction between mitoquinone and succinate or NADH in the absence of mitochondrial membranes, however mixing 50 mM mitoquinone, but not mitoquinol, with 50 mM ferricytochrome c led to some reduction of A550 ;

Figure 13 shows reactions of mitoquinol and mitoquinone with pentane-extracted mitochondrial membranes. Pentane extracted beef heart mitochondria (100 mg protein/ml) were suspended in 50 mM sodium phosphate, pH 7.2 at 20°C. In Panel A NADH (125 mM) was added and A_{340} was monitored and ubiquinone-1 (UQ-1; 50 mM) added where indicated. This was repeated in Panel B, except that mitoquinone (**1**) (50 mM) was added. In Panel C pentane extracted mitochondria were incubated with mitoquinone (**1**) (50 mM), A_{275} was monitored and succinate (5 mM) and malonate (20 mM) added where indicated. In Panel D pentane-extracted mitochondria were incubated with NADH (125 mM), ferricytochrome c (50 mM) and A_{550} was monitored and mitoquinone (**1**) (50 mM) was added where indicated. Addition of myxathiazol inhibited the rate of reduction by about 60-70%;

Figure 14 shows reduction of mitoquinone (**1**) by intact mitochondria. Rat liver mitochondria (100 mg/ml) were incubated in 120 mM KCl, 10 mM HEPES, 1 mM EGTA, pH 7.2 at 20°C. and A_{275} monitored. In Panel A rotenone and succinate (5 mM) were present and mitoquinone (**1**) (50 mM) was added where indicated. This experiment was repeated in the presence of malonate (20 mM) or FCCP (333 nM). In Panel B glutamate and malate (5 mM of each) were present from the start and mitoquinone (50 mM) was added where indicated. This experiment was repeated in the presence of FCCP or with rotenone and FCCP. Addition of TPMP (50 mM) instead of mitoquinone did not lead to changes in A_{275} ;

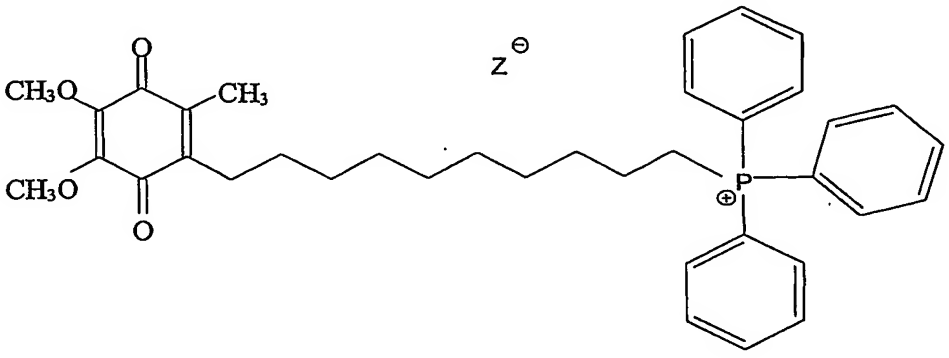
Figure 15 shows uptake of radiolabelled mitoquinol by energized rat liver mitochondria and its release on addition of the uncoupler FCCP; and

Figure 16 shows the effect of mitoquinol on isolated rat liver mitochondria. In Panel A rat liver mitochondria energized with succinate were incubated with various concentrations of mitoquinol and the membrane potential determined as a percentage of control incubations. In Panel B the respiration rate of succinate energized mitochondria under state 4 (black), state 3 (white) and uncoupled (stippled) conditions, as a percentage of control incubations.

EXAMPLE 5.

Antioxidant efficacy of exemplary mitochondrially-targeted compound mitoquinone/ol (compound 1)

A further exemplary compound is depicted in the following formula



Compound 1

which may exist as either the oxidized form (depicted) or as the quinol.

Stock solutions of the quinone in ethanol were stored at -80°C. in the dark and their concentrations confirmed by ^{31}P nmr. The compound was converted to the fully oxidized form by incubation in basic 95% ethanol over an hour on ice or by incubation with beef heart mitochondrial membrane at room temperature, either procedure leading to the same extinction coefficient of 10,400 M^{-1} cm^{-1} at the local maximum of 275 nm, with shoulders at 263 and 268 nm corresponding to the absorption maxima of the triphenylphosphonium moiety (Smith, R. A. J., Porteous, C. M., Coulter, C. V., and Murphy, M. P. 1999 *Eur J Biochem* **263**, 709. Burns *et al.*, 1995, *Arch Biochem Biophys* **332**, 60) and a broad shoulder at 290 nm due to the quinol (Crane et al, 1971 *Meth. Enzymol*, **18C**, 137). Reduction by addition of $NaBH_4$ gave the spectrum of the quinol which had the expected peak at 290 nm with an extinction coefficient of 1800 M^{-1} cm^{-1} and the extinction coefficient for at 268nm was 3,000 M^{-1} cm^{-1} the same as that for the phosphonium moiety alone (Burns *et al.*, 1995, *Arch Biochem Biophys* **332**, 60). The extinction coefficient of 10,400M^{-1} cm^{-1} at 275 nm was lower than that for other quinones which have values of 14,600 M^{-1} cm^{-1} in ethanol (Crane et al, 1971 *Meth. Enzymol*, **18C**, 137) and 12,250 M^{-1} cm^{-1} in aqueous buffer (Cabrini et al, 1981 *Arch Biochem Biophys*, **208**, 11). While the absorbance of the quinone was about 10% lower in buffer than in ethanol, the discrepancy was not due to an interaction between the phosphonium and the quinone as the absorbance of the precursor quinone before linking to the phosphonium and that of the simple phosphonium methyltriphenylphosphonium were additive when 50 mM of each were mixed together in either ethanol or aqueous buffer. The $\Delta\epsilon_{ox\text{-}red}$ was 7,000 M^{-1} cm^{-1}.

The spectrum of fully oxidized mitoquinone **(1)** (50 mM) in 50 mM sodium phosphate, pH 7.2 is shown in Figure 11. Addition of $NaBH_4$ gave the fully reduced compound, mitoquinol. The UV absorption spectrum of the reduced (quinol) and oxidized (quinone) forms of compound **(1)** are shown in Figure 11. To determine whether the mitochondrial respiratory chain could also oxidize or reduce compound **1** was incubated with beef heart mitochondrial membranes (Figure 12). In Panel A the spectrum of fully oxidized quinone form of compound **(1)** in the presence of antimycin inhibited membranes is shown (t=0; Figure 12A). Addition of succinate led to the gradual reduction of the quinol form of compound **(1)** as measured by repeating the measurement every five minutes and showing that the peak at 275 nm gradually disappeared, the presence of antimycin prevented the oxidation of the quinol by mitochondrial complex III. Succinate did not lead to the complete reduction of the quinone form of compound **(1)** to the quinol form, as can be seen by comparing the complete reduction brought about by borohydride (Figure 11), instead it

reduced about 23% of the added ubiquinone (Figure 12A). This is presumably due to equilibration of the quinol/quinone couple with the succinate/fumarate couple (Em Q=40 mV at pH 7, Em Suc=30 mV), hence this proportion corresponds to an Eh of about +8 mV.

The reduction of quinone form of compound (1) can be followed continuously at A_{275} nm (Figure 12B). On addition to rotenone inhibited mitochondrial membranes the small amount of the quinol form of compound (1) remaining was oxidized leading to a slight increase in A_{275}, but on addition of the Complex II substrate succinate the quinone form of compound (1) was rapidly reduced and this reduction was blocked by malonate, an inhibitor of Complex II (Figure 12B). The rate of reduction of the quinone form of compound (1) was 51+/-9.9 nmol/min/mg protein, which compares with the rate of reduction of cytochrome c by succinate in the presence of KCN of 359 nmol/min/mg. Allowing for the 2 electrons required for reduction of the quinone form of compound(1) compared with 1 for cytochrome c the rate of electron flux into the compound (1) pool is of similar order to the electron flux through the respiratory chain.

To determine whether the quinol form of compound (1) was oxidized by Complex III of the respiratory chain, the quinol form of compound (1) was added to beef heart membranes which had been inhibited with rotenone and malonate (Figure 12C). The quinol form of compound (1) was oxidized rapidly by membranes at an initial rate of about 89+/-9 nmol mitoquinol/min/mg protein (mean of 2+/-range) and this oxidation was blocked by myxathiazol an inhibitor of complex III (Figure 12C). To confirm that these electrons were being passed on to cytochrome c, the quinol form of compound (1) was then added to membrane supplemented with ferricytochrome c and the rate of reduction of cytochrome c monitored (Figure 12D). Addition of the quinol form of compound (1) led to reduction of cytochrome c at an initial rate of about 93+/-13 nmol/min/mg (mean +/-range). This rate was largely blocked by myxathiazol, although a small amount of cytochrome c reduction (about 30-40%) was not blocked by myxathiazol.

Compound (1) may be picking up and donating electrons directly from the active sites of the respiratory complexes, or it could be equilibrating with the endogenous mitochondrial ubiquinone pool. To address this question the endogenous ubiquinone pool was removed from beef heart mitochondria by pentane extraction. In the absence of endogenous ubiquinone as an electron acceptor the pentane extracted beef heart mitochondria could not oxidize added NADH, but addition of ubiquinone-1, a ubiquinone analog that can pick up electrons from the active site of complex I, the oxidation of NADH is partially restored

(Figure 13A). Similarly, addition of the quinone form of compound (**1**) also restored NADH oxidation indicating that the quinone form of compound (**1**) can pick up electrons from the complex I active site (Figure 13B). Succinate could also donate electrons to the quinone form of compound (**1**) in pentane extracted beef heart mitochondrial in a malonate sensitive manner, suggesting that the quinone form of compound (**1**) could also pick up electrons from the active site of Complex II (Figure 13C). Finally, the effect of the quinone on the flux of electrons to cytochrome c was determined and it was shown that there was no NADH-ferricytochrome c activity until the quinone form of compound (**1**) was added (Figure 13D), and this was partially inhibited by myxathizol (60-70%).

The next step was to see if the quinone form of compound (**1**) also accepted electrons within intact mitochondria (Figure 14). When the quinone form of compound (**1**) was added to intact energized mitochondria it was rapidly reduced (Figure 14A). In the presence of the uncoupler FCCP to dissipate the membrane potential the rate was decreased about 2-3 fold, presumably due to the prevention of the uptake of the compound in to the mitochondria (Figure 14A). The complex II inhibitor malonate also decreased the rate of reduction of the quinone form of compound (**1**) (Figure. 14A). Use of the NADH-linked substrates glutamate/malate also led to the rapid reduction of the quinone form of compound (**1**) by intact mitochondria which again was decreased by addition of the uncoupler FCCP (Figure 14B). The Complex I inhibitor rotenone also decreased the rate of reduction of the quinone form of compound (1) (Figure 14B).

A tritiated version of compound (**1**) was made in order to determine whether the quinol form was accumulated by energized mitochondria. This was incubated with energized mitochondria and the amount taken up into the mitochondria determined. It can be seen that the compound is accumulated rapidly and that this accumulation is reversed by addition of the uncoupler FCCP (Figure 15).

To determine the toxicity to isolated mitochondria and cells the effect on membrane potential and respiration rate were measured (Figure 16). It can be seen from Figure 16 that 10 mM mitoquinol had little effect on mitochondrial function and at 25 mM and above there was some uncoupling and inhibition of respiration.

EXAMPLE 6.

Antioxidant efficacy of exemplary mitochondrially-targeted compound (2)

The effect of compounds **1** or **2** on oxidative damage was assessed by measuring TBARS as described in Example 4 above and shown in Figure 4. Briefly, Rat liver mitochondria (10 mg protein) were incubated at 25°C in a shaking water bath in 2 ml medium containing 100 mM KCl, 10 mM Tris, pH 7.7, supplemented with rotenone (1.33 μg/ml), 10 mM succinate, 500 μM ascorbate and other additions. After preincubation for 5 min, 50 μM $FeCl_2$ was added and 45-55 min later duplicate samples were removed and assayed for TBARS fluorometrically (λ_{excite} = 515 nm; $\lambda_{emission}$ = 553 nm).

Isolated mitochondria were exposed to oxidative stress by incubation with iron/ascorbate. As can be seen in Figure 17, the antioxidant efficiency of compound (**2**) is in the same order as that observed for compound (**1**), thereby demonstrating the antioxidant efficacy of the antioxidant compounds of the present invention.

EXAMPLE 7

Effect of mitochondrially targeted antioxidant compounds on cardiac haemodynamics and mitochondrially function.

The effect of administration of mitochondrially targeted antioxidant compounds, in particular compound **1** and compound **2**, on cardiac function was assessed using the Langendorf isolated heart perfusion model. Rats were assigned to the following four administration groups: Control (placebo), TPMP (methyltriphenyl phosphonium), compound **1**, and compound **2**. Following the treatment period, rats were humanely sacrificed and the isolated hearts were connected to the Langendorf isolated perfusion system. This system uses retro-perfusion through the aorta to maintain the heart while cardiac function is measured. Left ventricular pressure was measured with a left ventricular balloon. Coronary flow was also measured.

Figure 18 depicts the coronary flow at 10 mmHg left ventricular pressure for each of the treatment groups. Coronary flow was measured pre-ischaemia and again at zero minutes, 20 minutes, 40 minutes and 60 minutes following induction of ischaemia. A one way ANOVA with bonferroni post hoc test was performed. Significance versus pre-ischaemic control: * $P<0.05$; ** $P<0.01$; *** $P<0.001$. Significance versus respective time control: † $P<0.05$; †† $P<0.01$; ††† $P<0.001$.

The results show that treatment with compound **1** significantly reduces the ischaemia-induced reduction in coronary flow. Compound **2** has a lesser but still significant effect at the

later time points. The absence of any effect with administration of TPMP indicate that it is the antioxidant moiety of compound **1** and compound **2**, and not the triphenylphosphonium cation, that is responsible for the effects observed with the mitochondrially targeted antioxidant compounds.

Figure 19 depicts the effects of treatment on left ventricular diastolic pressure at 10 mmHg. Left ventricular diastolic pressure was measured prior to induction of ischaemia and again at zero minutes, 20 minutes, 40 minutes and 60 minutes following the induction of ischaemia. Statistical analysis was an ANOVA on ranks with Dunns post hoc test. Significance verses pre-ischaemic control: * $P<0.05$. † represents $P<0.05$ versus 60 min post ischaemic control. The results show that treatment with compound **1** results in a statistically significant increase in left ventricular diastolic pressure verses untreated rats, reducing the ischaemia-induced reduction in left ventricular diastolic pressure. The absence of any effect with administration of TPMP indicate that it is the antioxidant moiety of compound **1**, and not the triphenylphosphonium cation, that is responsible for the effects observed with the mitochondrially targeted antioxidant compounds.

The effect of administration of compound **1** and compound **2** on heart rate was then determined. Figure 20 depicts the heart rate for each of the treatment groups pre-ischaemia, and zero minutes, 20 minutes, 40 minutes and 60 minutes following the induction of ischaemia. Results shown are one way ANOVA followed by bonferroni post hoc test. *** represents $P<0.001$ versus pre-ischaemic control. †† represents $P<0.05$ versus respective post ischaemic control. The results show that treatment with compound **1** significantly reduces the ischaemia induced reduction in heart rate compared to control rats. The absence of any effect with administration of TPMP indicate that it is the antioxidant moiety of compound **1**, and not the triphenylphosphonium cation, that is responsible for the effects observed with the mitochondrially targeted antioxidant compounds.

Cardiac function was further assessed by determining the effect of administration of mitochondrially targeted antioxidant compounds on the rate of contraction and relaxation of the heart. Figure 21A depicts the rate of contraction in each of the four treatment groups pre-ischaemia, and zero minutes, 20 minutes, 40 minutes and 60 minutes following induction of ischaemia. Figure 21B depicts the rate of relaxation in each of the four treatment groups pre-ischaemia, and zero minutes, 20 minutes, 40 minutes and 60 minutes following induction of ischaemia. In each case ANOVA was performed on ranks with Dunns post hoc test performed. * represents significance with $P<0.05$ verses pre-ischaemia control. † represents

significance with $P<0.05$ versus respective post ischaemic time controls. †† represents significance with $P<0.01$ versus respective post ischaemic time control.

The results show that administration of compound **1** has a statistically significant effect, reducing the ischaemia-induced reduction in the rate of contraction and relaxation of the left ventricle when compared to control rats.

The above data clearly show the beneficial effect of administration of mitochondrially targeted antioxidant compounds on cardiac function. In order to determine whether the observed effects on cardiac function were due to the effect of the mitochondrial targeted antioxidant compound on mitochondrial function, mitochondrial activity pre-ischaemia and post-ischaemia was assessed for each of the treatment groups. Figure 22A depicts NAD^+ linked respiratory function of mitochondria pre and post-ischaemia for each of the four treatment groups. Figure 22B presents FAD linked respiratory function pre and post-ischaemia for each of the four treatment groups. *** represents significance with $P<0.001$ versus pre-ischaemic control. ††† represents significance with $P<0.001$ versus post ischaemic control.

These data show that compound **1** has a statistically significant beneficial effect on mitochondrially respiratory function following ischaemia compared to control rats. These results support the conclusion that the effects of administration of mitochondrially targeted antioxidant compounds on cardiac function is due to a protective effect on mitochondrial function.

EXAMPLE 8

Stability of compound 1 complexes with β–cyclodextrin

In preformulation studies compound **1** as the bromide salt was found to degrade over time in the solid state when stored at 25°C, 50% RH and 40°C, 75%RH. The objective of the present study was to establish whether the solid state stability of compound **1** could be improved by complexing with β–cyclodextrin.

Compound **1** bromide (batch no. 3 and batch no. 4) was kindly supplied by Dr. Rob Smith of the Chemistry Department. Compound **1** batch no. 6 and idebenone were supplied by Industrial Research Limited (New Zealand). β–cyclodextrin (lot no. 70P225) was purchased from ISP technologies Inc. NaCI, NaH PU and methanol (HPLC) were purchased from BDH.

Solid state stability study of pure compound 1

Samples of compound **1** (approximately 5 mg) were accurately weighed into clear bottles and exposed to 25°C, 50%RH, 40°C, 75%RH and 4°C over silica. The bottles were removed after 1, 2, 4, 8, 16, 32 and 64 days and analysed for compound **1** by a validated HPLC method using compound **1** stored at -20°C over silica as control.

Preparation of compound 1: β–cyclodextrin complexes

Three complexes with different molar ratios (compound **1** bromide: β–cyclodextrin 1:1, 1:2 and 1:4) were prepared using compound **1** batch no. 6.

Preparation of β–cyclodextrin solution in water

β–cyclodextrin (1.1397 g, equal to 1.0361 g after correction for moisture content) was accurately weighed and dissolved in double distilled water by sonicating for 10 min. The volume was made up to 100 ml with water.

Preparation of compound 1: β–cyclodextrin (1:1 molar ratio) complex

An ethanolic solution of compound **1** bromide (90 mg equal to 59.95 mg of compound **1**) was evaporated under nitrogen on a hot plate maintained at 40-50°C for 8 min. β–cyclodextrin solution (10 ml) and double distilled water (30ml) were added to the beaker which was then sonicated for 40 min.

Preparation of compound 1: β–cyclodextrin (1:2 molar ratio) complex

An ethanolic solution of compound **1** bromide (89.8 mg equal to 59.82 mg of compound **1**) was evaporated under nitrogen on a hot plate maintained at 37 - 45°C for 10 min followed by 3 min at 50°C. β–cyclodextrin solution (20 ml) and double distilled water (20 ml) were added to the beaker which was then sonicated for 30min.

Preparation of compound 1: β–cyclodextrin (1:4 molar ratio) complex

An ethanolic solution of compound **1** bromide (90mg equal to 59.95 mg of compound **1**) was evaporated under nitrogen on a hot plate maintained at 37-50°C for 12 min. β–cyclodextrin solution (40 ml) was added to the beaker which was then sonicated for 20 min.

All the above solutions were frozen by storing at -18°C overnight. The frozen solutions were freeze-dried for 2 days using the LABCONO freeze drier. The lyophilized compounds were stored at -20°C.

DSC of the freeze-dried compound 1 : β–cyclodextrin complexes

Differential scanning calorimetry of the three freeze-dried complexes was carried out using a Perkin Elmer Differential Scanning Calorimeter PYRIS- 1. A compound **1** sample was prepared by evaporating an ethanolic solution under nitrogen gas at 35 - 50°C for 10 min.

Aluminium pans (No.0219-0041, supplied by Perkin-Elmer) were used. The analysis was carried out under nitrogen purge. Empty pans were used to set the baseline.

Scanning temperature range was 50-160°C with an initial hold at 50°C for 1min followed by an increase of 10°C/min up to 160°C.

HPLC assay

An HPLC method for compound **1** was developed using methanol and 0.01M sodium dihydrogen phosphate (85:15) as mobile phase at a flow rate of 1 ml/min and using UV-VIS detection at 265 nm. The internal standard was idebenone. The column was Prodigy ODS3100A (Phenomenex) with particle size 5μ. Later this method was modified after the arrival of a new column. The mobile phase used in the modified method was methanol and 0.01M sodium dihydrogen phosphate (80:20). This method was validated. Interference by β–cyclodextrin in the HPLC method was checked before analysing the compound **1**: β–cyclodextrin complexes. It was shown that β–cyclodextrin does not interfere in the compound **1** HPLC assay.

Stability study of compound 1: β–cyclodextrin complexes

As there were three complexes of compound **1** with β–cyclodextrin, the amount of compound **1** in 5 mg samples from the different complexes was different. In order to expose equal amounts of compound **1** in all three complexes, different weights of complexes were taken: 4 mg of 1:1 complex containing 1.473 mg of compound **1**; 6.5 mg of 1:2 complex containing 1.469 mg of compound **1**; and 11.5 mg of 1:4 complex containing 1.467 mg of compound **1** were taken and used in the stability study as per the Standard Operating Procedure.

Aliquots of HPLC water (1.5 ml) were added to each sample bottle to completely dissolve the compound **1**: β–cyclodextrin complexes. Aliquots (50 μl) of these solutions were diluted to 1 ml with water. Aliquots (100 μl) of these diluted solutions of compound **1**: β–cyclodextrin complexes were vortexed with 200 μl of a 10 μg/ml solution of internal standard in methanol. The samples were centrifuged for 10 min at 10000 rpm and 50 μl of the

supernatants injected into the HPLC system. A standard curve was prepared using solutions of compound **1** in the concentration range 2.5 to 120 μg/ml containing 5 mg/ml solutions of β–cyclodextrin.

All the compounds were slighty orange-yellow in colour and very fluffy in appearance. The colour was not uniform and was more concentrated towards the bottom of the freeze drying flasks.

The results of DSC are given as follows:

Compound 1: When a pure sample of compound **1** was analyzed, peaks were observed above 120°C. With one sample of compound **1**, two prominent peaks were observed between 130°C and 140°C. When another sample was analyzed, no such prominent peaks were observed but small peaks were observed above 120°C. After analysis, the pans were cut and the samples examined. The samples were dark green to black in colour in both cases.

β–cyclodextrin: There was a broad peak between 70°C and 85°C.

Compound 1: β–cyclodextrin (1:1) complex: No significant peaks were observed. After analysis the pan was cut and examined. The sample colour had undergone a slight change to light brown (not a significant change).

Compound 1: β–cyclodextrin (1:2) complex: No significant peaks were observed. After analysis, no colour change in the sample was observed.

Compound 1: β–cyclodextrin (1:4) complex: No significant peaks were observed but a very small exothermic peak was observed at 120°C. After analysis, no colour change in the sample was observed.

The appearance of peaks in the compound 1 pure sample indicates that changes in the compound are taking place with temperature. However, as there were many peaks and also colour changes in the sample, these could have arisen due to degradation. When a second sample of compound **1** was analyzed, it gave a different thermogram to the first sample. In the case of the complexes, there were no significant peaks or any colour changes.

The results of the solid state stability study of pure compound **1** (batch no. 3) are given in Table 3and Figure 23.

Table 3. Solid state stability of compound **1** (batch no. 3).

Clear Glass Bottles	Day 1	Day 2	Day 4	Day 8	Day 16	Day 32	Day 64

40°C, 75%RH	98.90	101.9	102.8	94.07	83.22	76.70	67.25
25°C, 50%RH	95.11	97.46	95.06	97.52	102.8	40.76	18.37
5°C, silica gel	97.04	102.8	92.97	95.67	98.37	67.36	63.70

Solid state stability of compound **1** (batch no. 3) in the absence of light at 40°C, 75%RH; 25°C, 50%RH and 5°C over blue silica gel. Data are means of two values expressed as percentage of orginal content.

Due to the significant instability at 25°C, 50%RH compared to 40°C, 75%RH, the stability study was repeated at 25°C, 50% RH with compound **1** batch no. 4. The second stability study was conducted both in clear and amber bottles and the results are given in Table 4and Figure 24.

Table 4. Solid state stability of compound **1** (batch no. 4)

Time (days)	1	2	4	8	16	32	64
Clear Glass Bottles	88.21	93.19	92.65	93.10	94.47	62.05	57.94
Dark Amber Glass Bottles	94.84	94.52	100.28	97.65	98.03	61.48	58.66

Solid state stability of compound **1** (batch no. 4) was measured in the absence of light at 25°C, 50 %RH. Data are means of three values expressed as percentage of the initial content.

Both batches (batches no.3 and 4) of compound **1** supplied by the Chemistry Department showed a sudden drop in content after 16 days. However, for batch no. 4 the degradation was not as great after 32 to 64 days compared to batch no. 3. Also it was observed whether the bottles were clear or amber had no effect on compound **1** stability.

The compound **1** supplied from IRL was used for the preparation of the compound **1**: β–cyclodextrin complexes. The compound **1** supplied from IRL was a reddish-yellow syrup in ethyl alcohol. The stability of the compound **1**: β–cyclodextrin complexes is given in Table 5 and in Figures 25, 26 and 27. Because of the small amounts of compound **1**: β–cyclodextrin complexes available for study, there are no results for day 1 and day 4.

Table 5. Solid state stability of compound **1**: β–cyclodextrin complexes

1:1 complex					

Time (days)	2	8	16	32	64
4°C, silica	106.38	110.97	101.71	101.71	102.68
25°C,50%RH	95.65	93.00	101.15	101.15	108.89
40°C,75%RH	129.22	108.77	113.48	113.49	89.25
1:2 complex					
Time (days)	**2**	**8**	**16**	**32**	**64**
4°C,silica	105.48	101.23	105.08	111.21	101.16
25°C,50%RH	108.16	95.46	105.41	108.55	99.78
40°C,75%RH	115.99	110.22	114.03	101.50	99.44
1:4 complex					
Time (days)	**2**	**8**	**16**	**32**	**64**
4°C, silica	105.10	115.86	100.25	107.63	107.63
25°C, 50%RH	111.46	116.03	96.61	92.40	92.40
40°C, 75%RH	108.85	100.01	87.34	71.13	71.13

Solid state stability of compound 1: β–cyclodextrin complexes in the absence of light at 40°C, 75%RH; 25°C, 50%RH and 5°C over blue silica gel. Data are means of two values expressed as percentage.

The results show that compound **1** can effectively form complexes with β–cyclodextrin, and can be stabilized by complexing with β–cyclodextrin. The results show that compound **1** in the 1:1 and 1:2β–cyclodextrin complexes was stable under various conditions. The results also show that the stability of compound **1** in the 1:4 complex was decreased relative to the stability of compound **1** in the 1:1 and 1:2 β–cyclodextrin complexes.

***

All patents, publications, scientific articles, and other documents and materials referenced or mentioned herein are indicative of the levels of skill of those skilled in the art to which the invention pertains, and each such referenced document and material is hereby incorporated by reference to the same extent as if it had been incorporated by reference in its entirety individually or set forth herein in its entirety. Applicants reserve the right to physically incorporate into this specification any and all materials and information from any such patents, publications, scientific articles, web sites, electronically available information, and other referenced materials or documents.

The specific methods and compositions described herein are representative of various embodiments or preferred embodiments and are exemplary only and not intended as limitations on the scope of the invention. Other objects, aspects, examples and embodiments will occur to those skilled in the art upon consideration of this specification, and are encompassed within the spirit of the invention as defined by the scope of the claims. It will be readily apparent to one skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention. The invention illustratively described herein suitably may be practiced in the absence of any element or elements, or limitation or limitations, which is not specifically disclosed herein as essential. Thus, for example, in each instance herein, in embodiments or examples of the present invention, any of the terms "comprising", "consisting essentially of", and "consisting of" may be replaced with either of the other two terms in the specification. Also, the terms "comprising", "including", containing", etc. are to be read expansively and without limitation. The methods and processes illustratively described herein suitably may be practiced in differing orders of steps, and that they are not necessarily restricted to the orders of steps indicated herein or in the claims. It is also that as used herein and in the appended claims, the singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, a reference to "a host cell" includes a plurality (for example, a culture or population) of such host cells, and so forth. Under no circumstances may the patent be interpreted to be limited to the specific examples or embodiments or methods specifically disclosed herein. Under no circumstances may the patent be interpreted to be limited by any statement made by any Examiner or any other official or employee of the Patent and Trademark Office unless such statement is specifically and without qualification or reservation expressly adopted in a responsive writing by Applicants.

The terms and expressions that have been employed are used as terms of description and not of limitation, and there is no intent in the use of such terms and expressions to exclude any equivalent of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention as claimed. Thus, it will be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the appended claims.

The invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or

negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

Other embodiments are within the following claims. In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group.

The compounds of the invention have application in selective antioxidant therapies for human patients to prevent mitochondrial damage. This can be to prevent the elevated mitochondrial oxidative stress associated with particular diseases, such as Parkinson's disease or diseases associated with mitochondrial DNA mutations. They could also be used in conjunction with cell transplant therapies for neurodegenerative diseases, to increase the survival rate of implanted cells.

In addition, these compounds could be used as prophylactics to protect organs during transplantation, or ameliorate the ischaemia-reperfusion injury that occurs during surgery. The compounds of the invention could also be used to reduce cell damage following stroke and heart attack or be given prophylactically to premature babies, which are susceptible to brain ischemia. The methods of the invention have a major advantage over current antioxidant therapies – they will enable antioxidants to accumulate selectively in mitochondria, the part of the cell under greatest oxidative stress. This will greatly increase the efficacy of antioxidant therapies. Related lipophilic cations are being trialed as potential anticancer drugs and are known to be relatively non-toxic to whole animals, therefore these mitochondrially-targeted antioxidants are unlikely to have harmful side effects.

Those persons skilled in the art will appreciate that the above description is provided by way of example only, and that different lipophilic cation/antioxidant combinations can be employed without departing from the scope of the invention.

DATED THIS 23d DAY OF October 20[illegible]
AJ PARK
PER Hamdale
AGENTS FOR THE APPLICANT

Figure 1

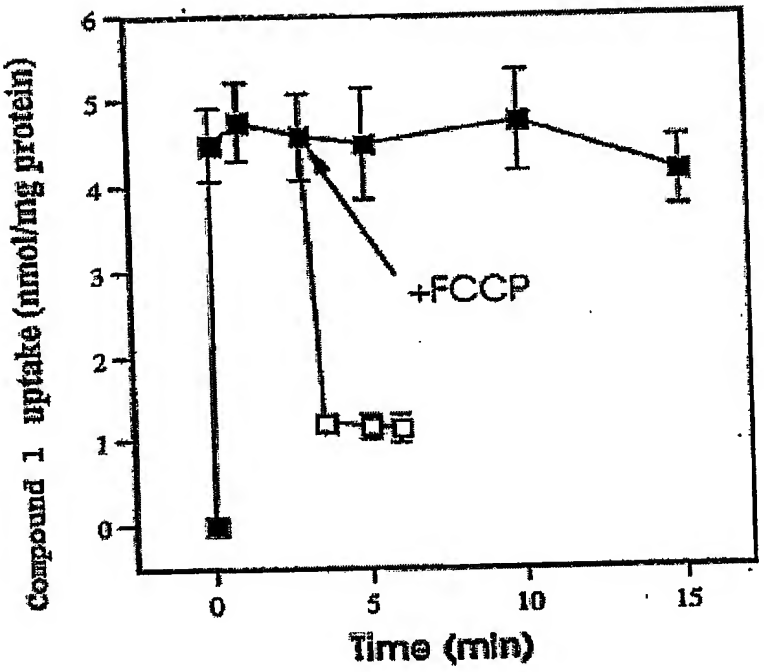

Compound 1 uptake (nmol/mg protein)
6
5
4
3
2
1
0
+FCCP
0
5
10
15
Time (min)

Figure 2

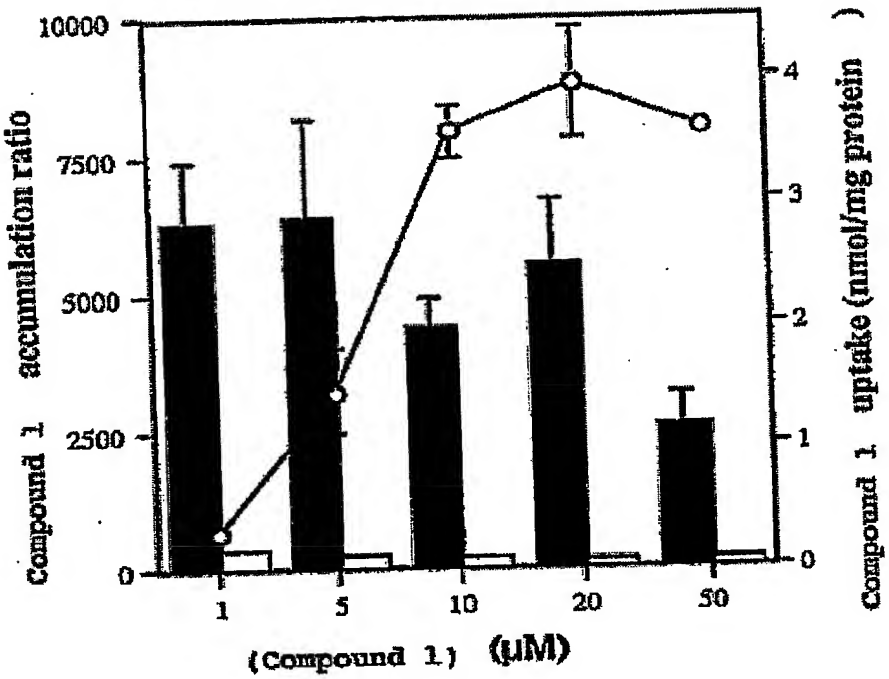

10000
7500
5000
2500
0
Compound 1 accumulation ratio
4
3
2
1
0
Compound 1 uptake (nmol/mg protein
1
5
10
20
50
(Compound 1) (μM)

Figure 3

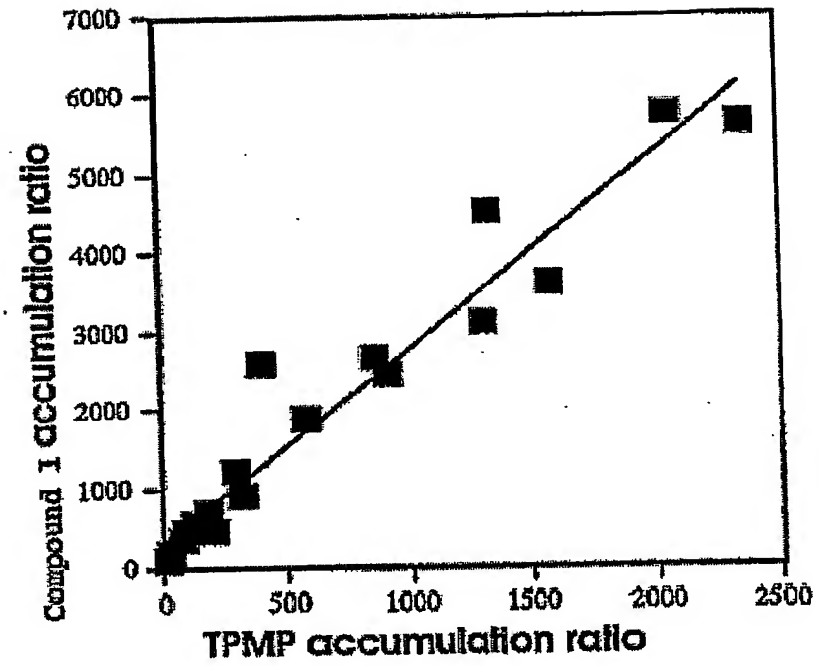

7000
6000
5000
4000
3000
2000
1000
0
Compound 1 accumulation ratio
0
500
1000
1500
2000
2500
TPMP accumulation ratio

Figure 4

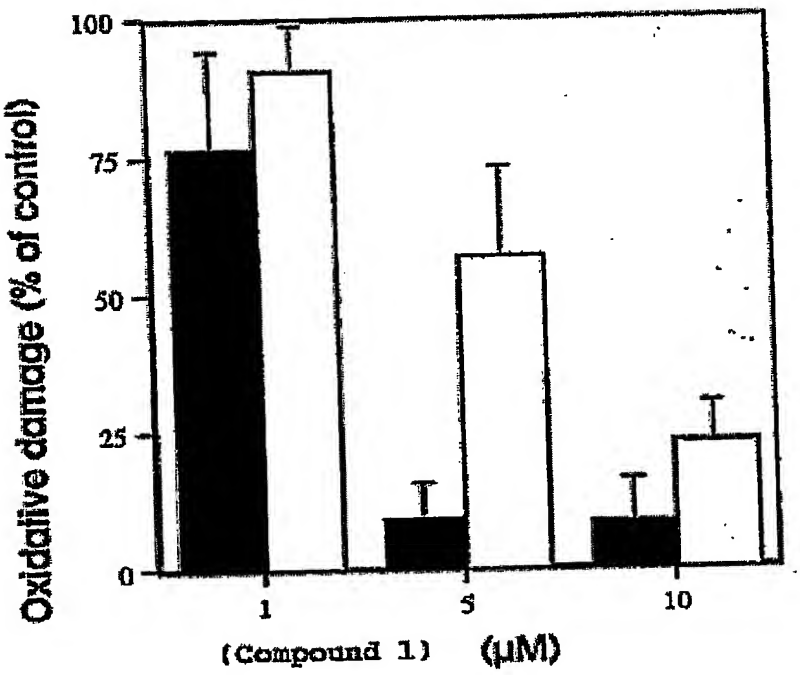


Figure 5

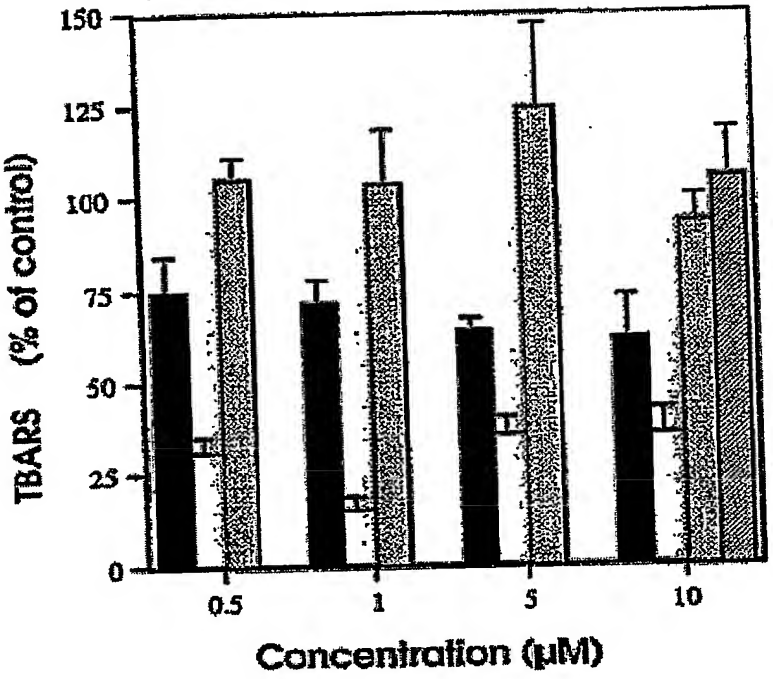

150
125
100
75
50
25
0
TBARS (% of control)
0.5
1
5
10
Concentration (μM)

Figure 6

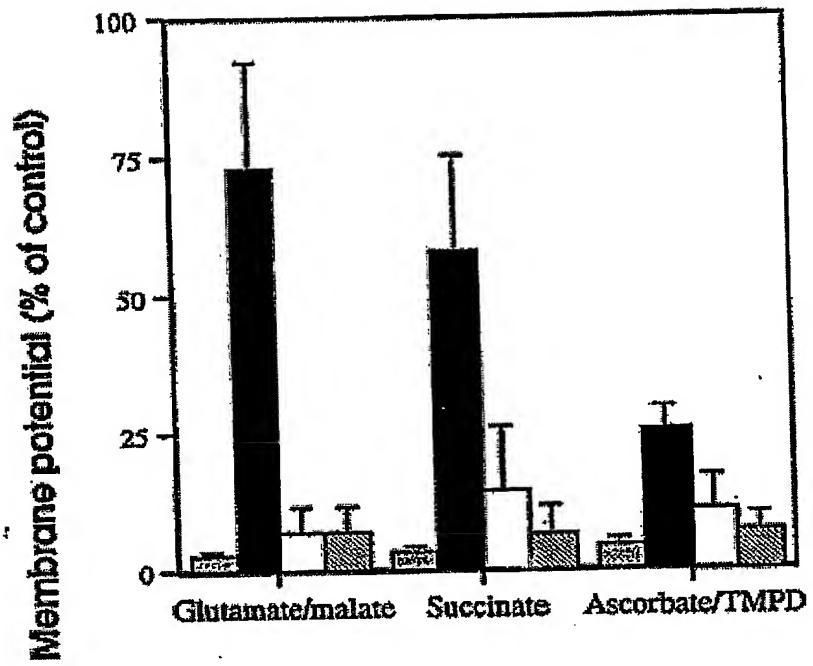

Membrane potential (% of control)
100
75
50
25
0
Glutamate/malate
Succinate
Ascorbate/TMPD

Figure 7

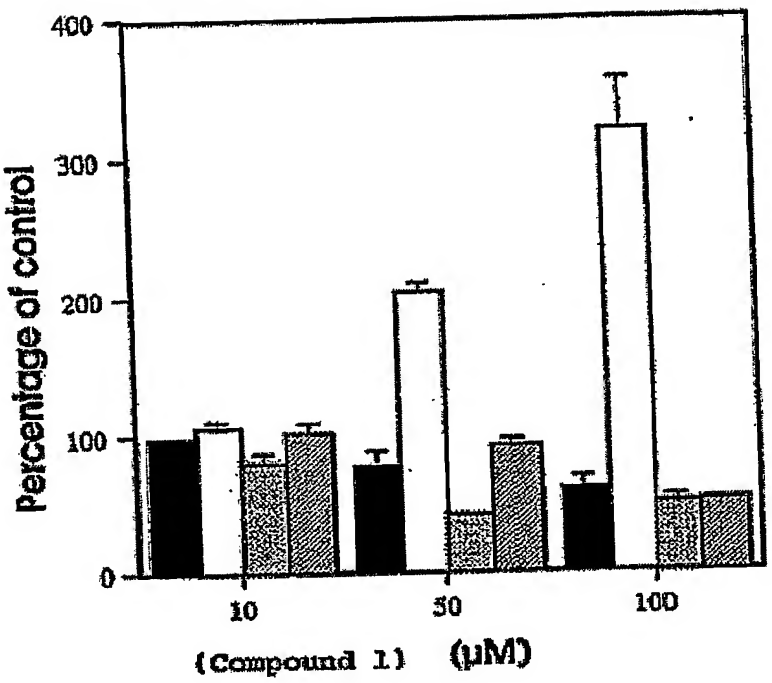

400
300
200
100
0
Percentage of control
10
50
100
(Compound 1) (μM)

Figure 8

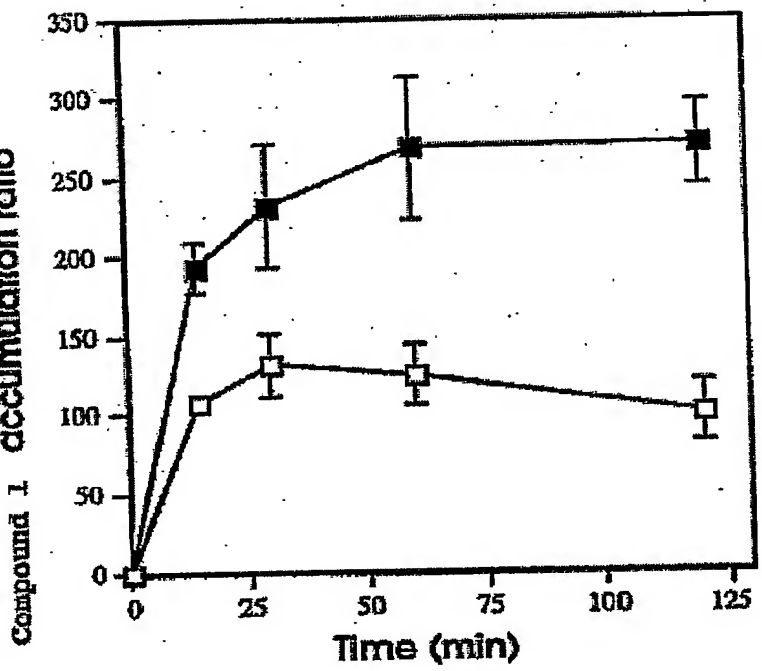

Compound 1 accumulation ratio
350
300
250
200
150
100
50
0
0
25
50
75
100
125
Time (min)

Figure 9

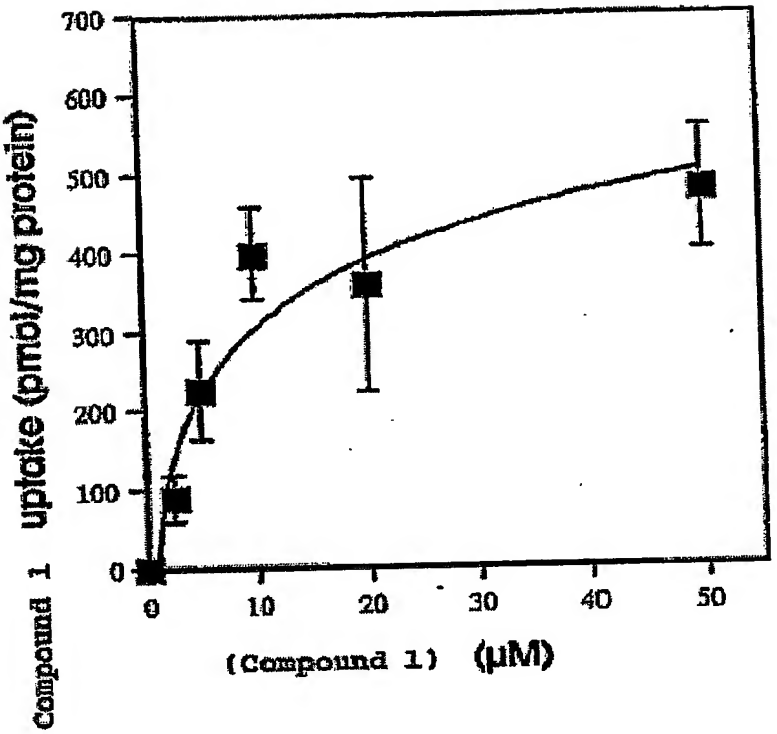

700
600
500
400
300
200
100
0
Compound 1 uptake (pmol/mg protein)
0
10
20
30
40
50
(Compound 1) (μM)

Figure 10

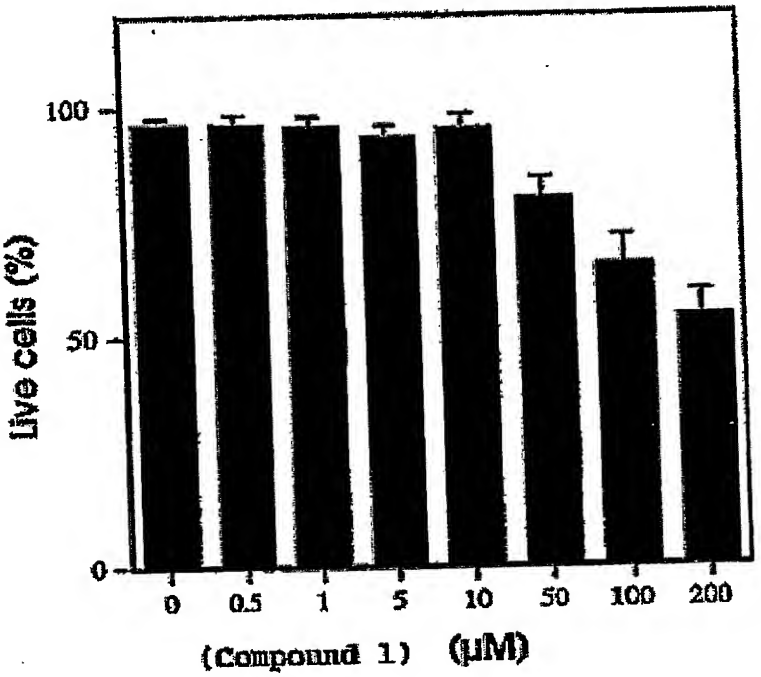


Figure 11

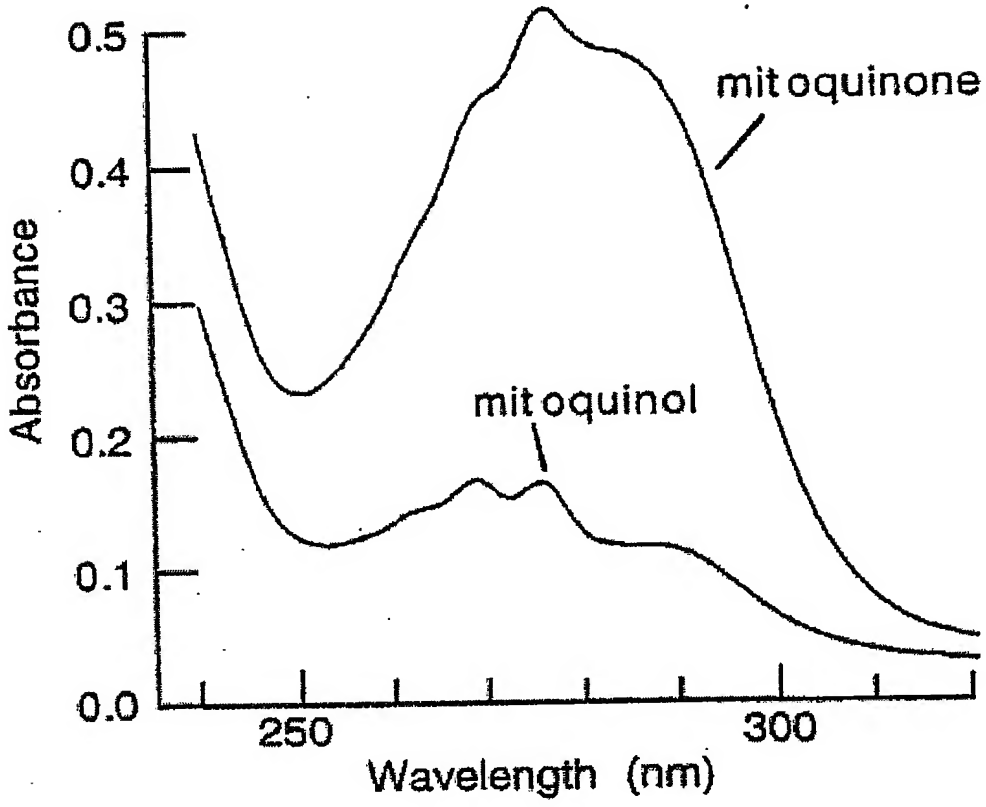

0.5
0.4
0.3
0.2
0.1
0.0
Absorbance
mitoquinone
mitoquinol
250
300
Wavelength (nm)

Figure 12

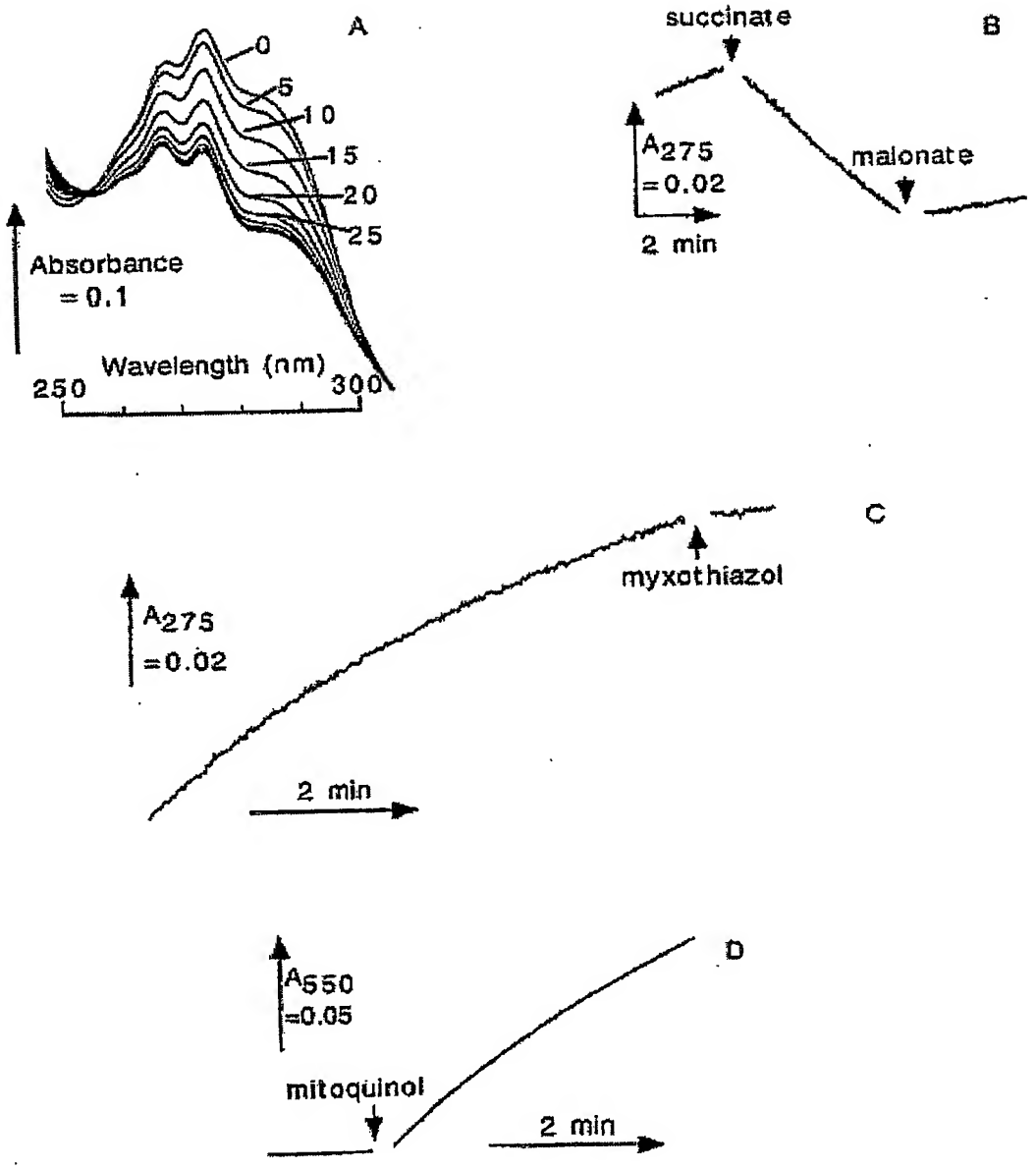

A
0
5
10
15
20
25
Absorbance
=0.1
Wavelength (nm)
250
300
succinate
B
A_{275}
=0.02
malonate
2 min
C
myxothiazol
A_{275}
=0.02
2 min
D
A_{550}
=0.05
mitoquinol
2 min

Figure 13

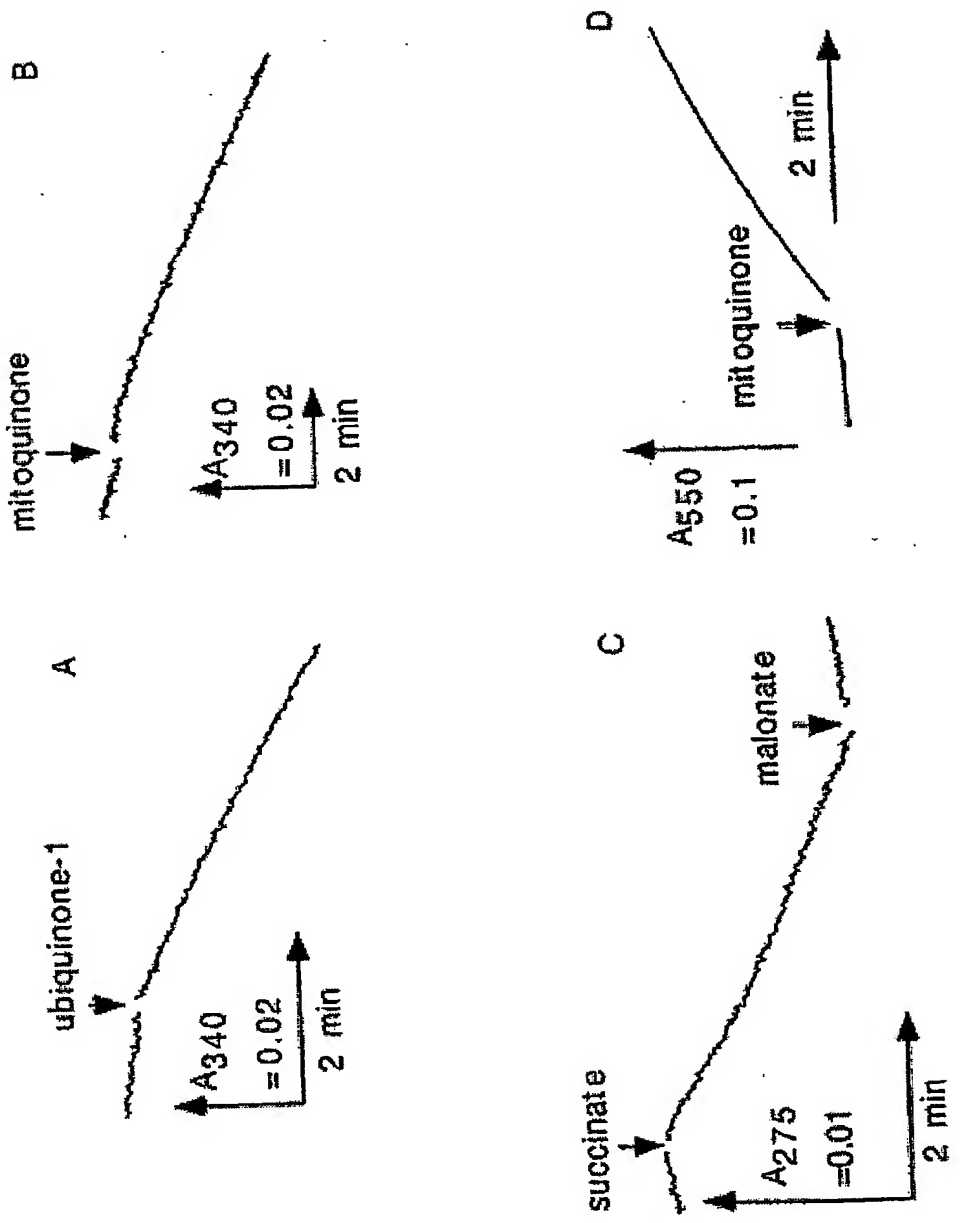

A
ubiquinone-1
A340
=0.02
2 min
B
mitoquinone
A340
=0.02
2 min
C
malonate
succinate
A275
=0.01
2 min
D
mitoquinone
A550
=0.1
2 min

Figure 14

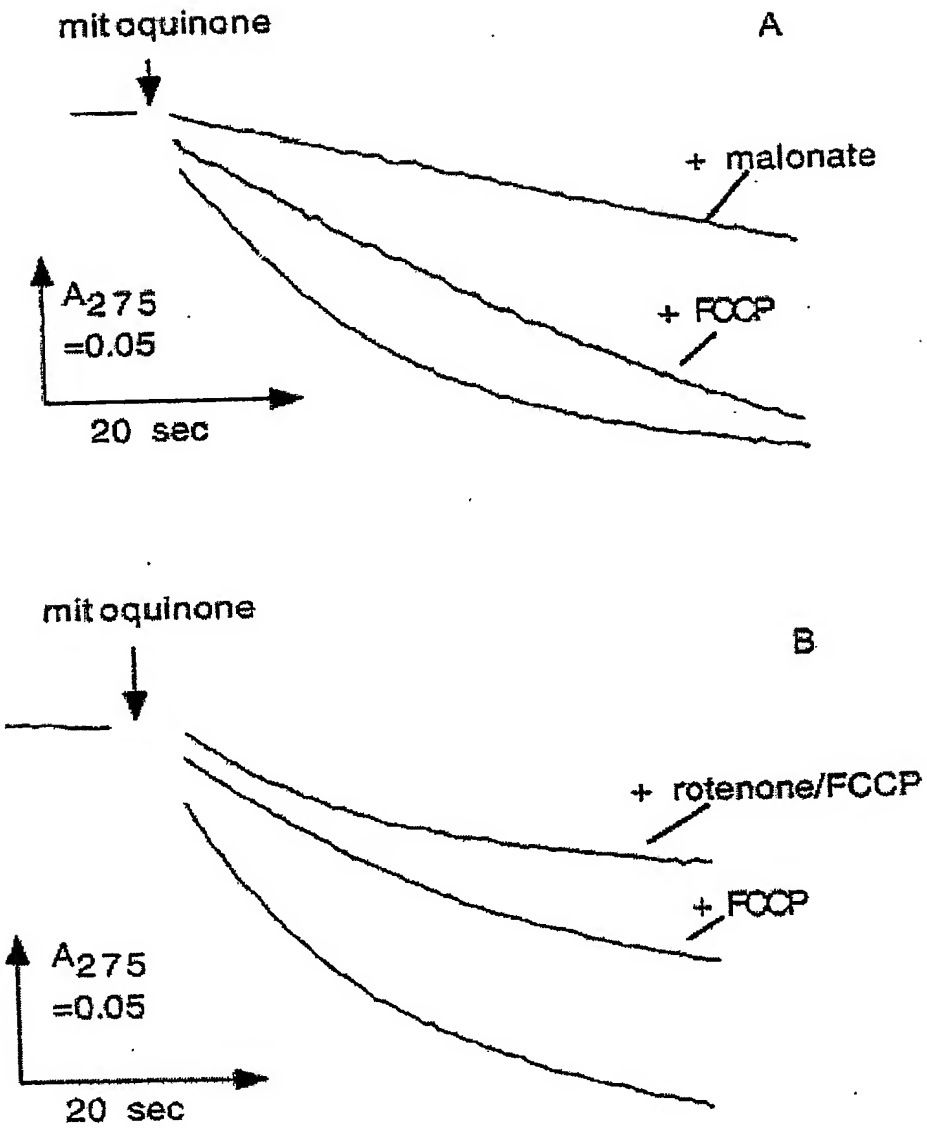

mitoquinone
A
+ malonate
+ FCCP
A_{275}
=0.05
20 sec
mitoquinone
B
+ rotenone/FCCP
+ FCCP
A_{275}
=0.05
20 sec

Figure 15

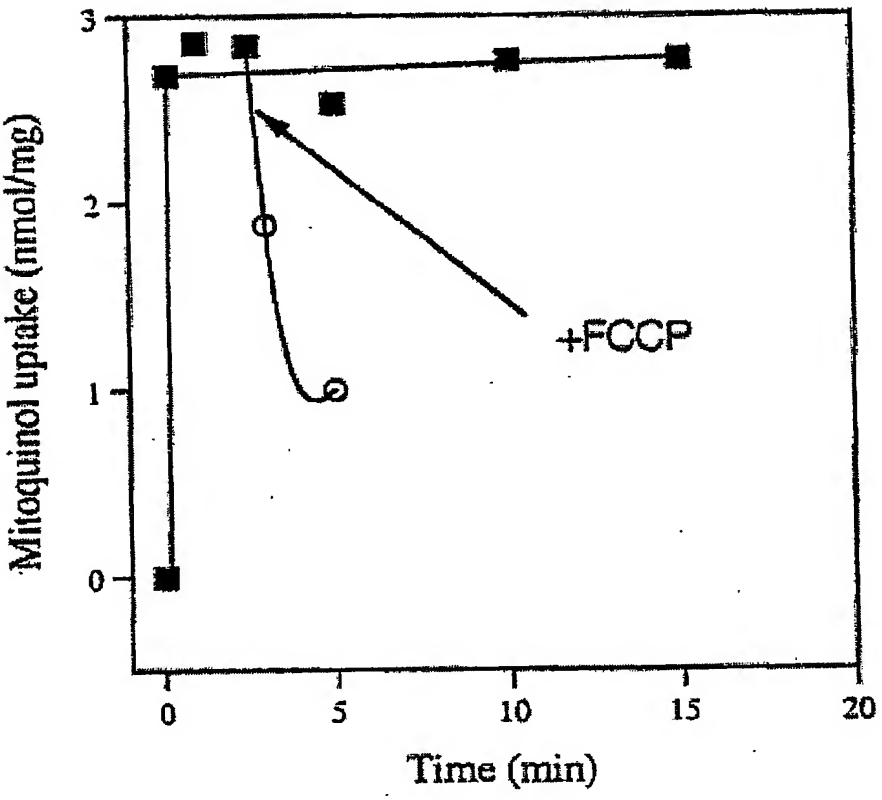


Figure 16A

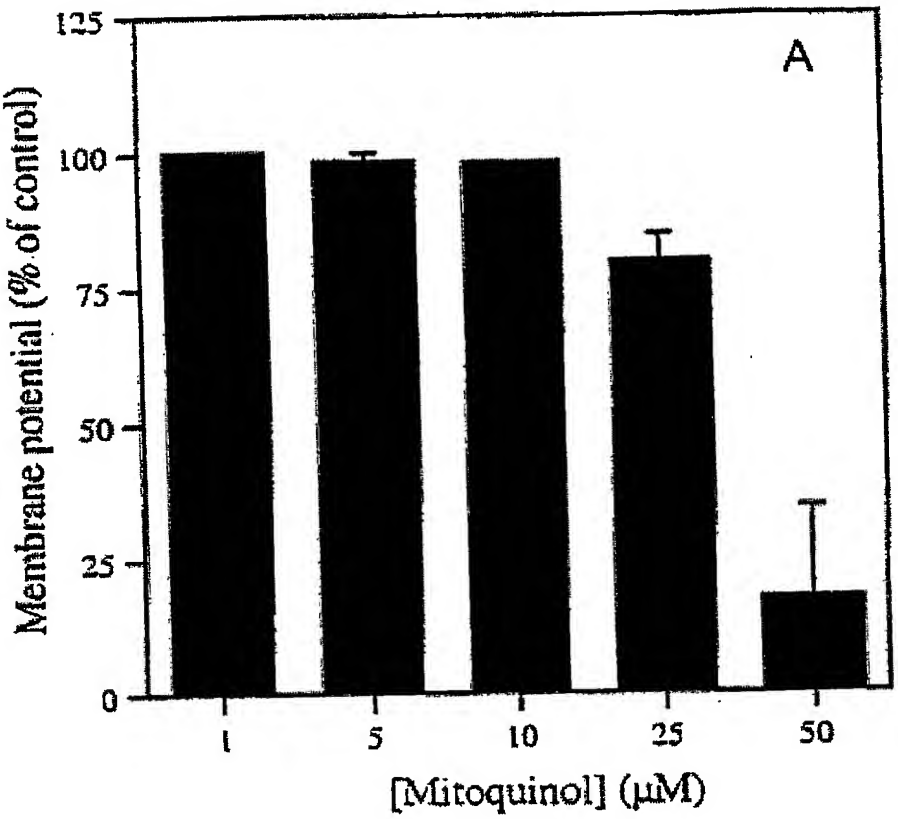
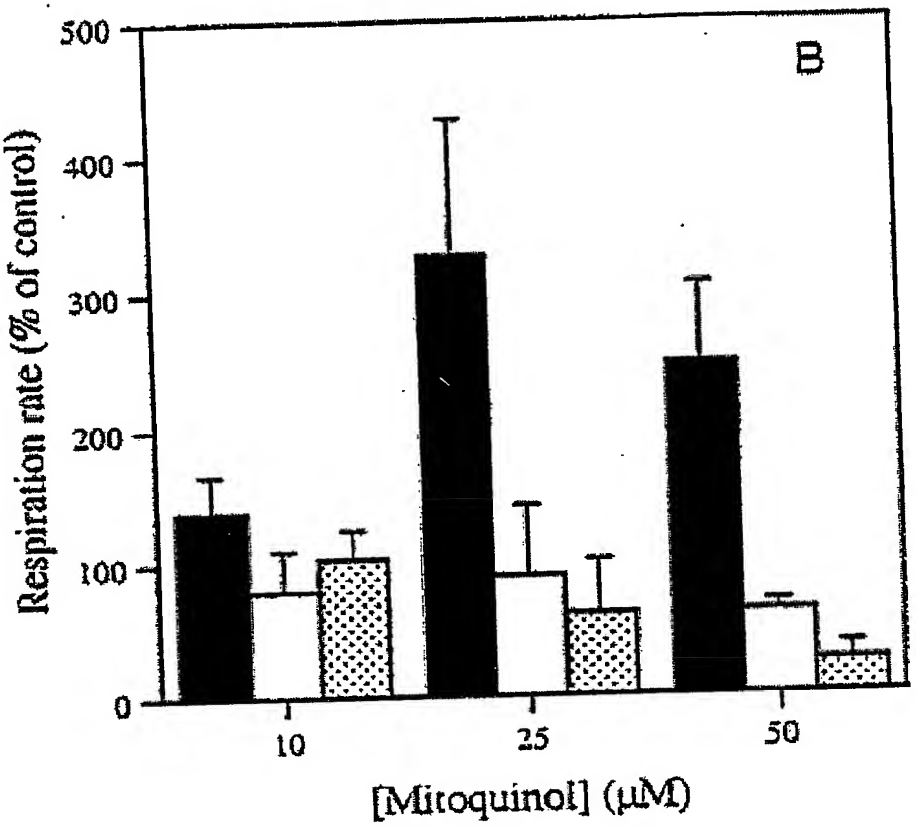

A
125
100
75
50
25
0
Membrane potential (% of control)
1
5
10
25
50
[Mitoquinol] (μM)

Figure 16B


B
500
400
300
200
100
0
Respiration rate (% of control)
10
25
50
[Mitoquinol] (μM)

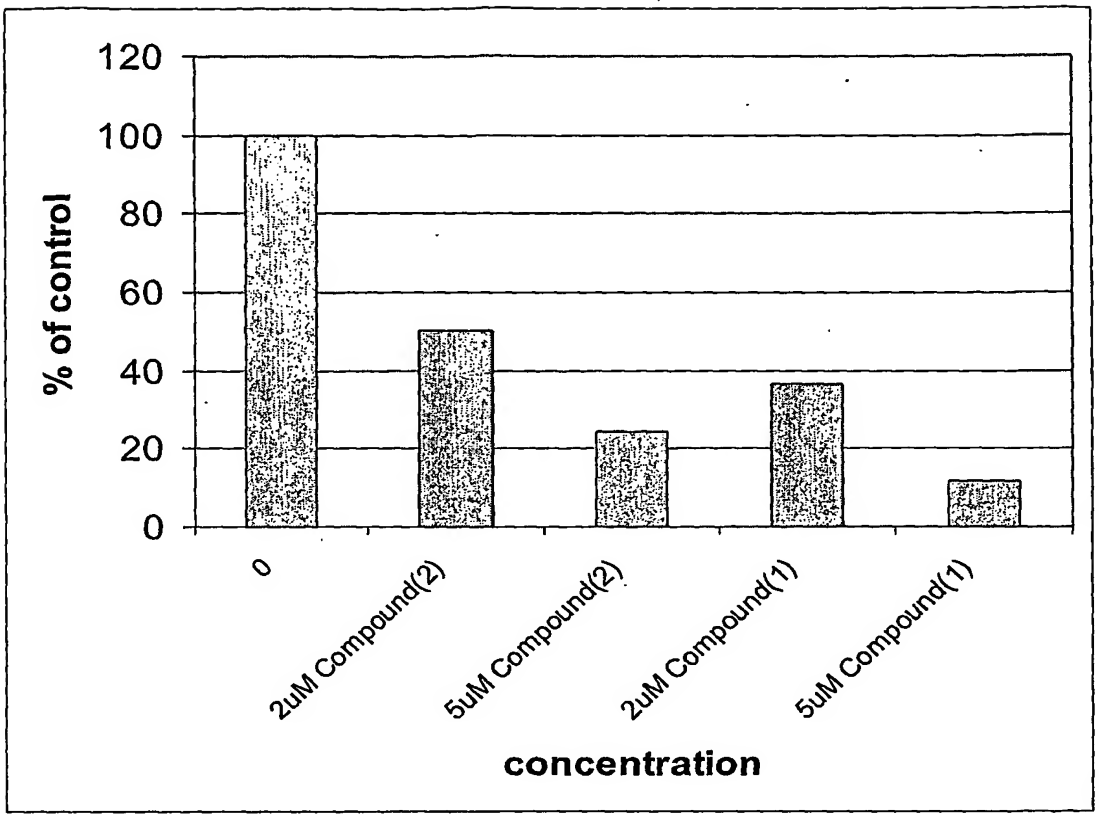

120
100
80
60
40
20
0
% of control
0
2uM Compound(2)
5uM Compound(2)
2uM Compound(1)
5uM Compound(1)
concentration

Figure 17

Figure 18

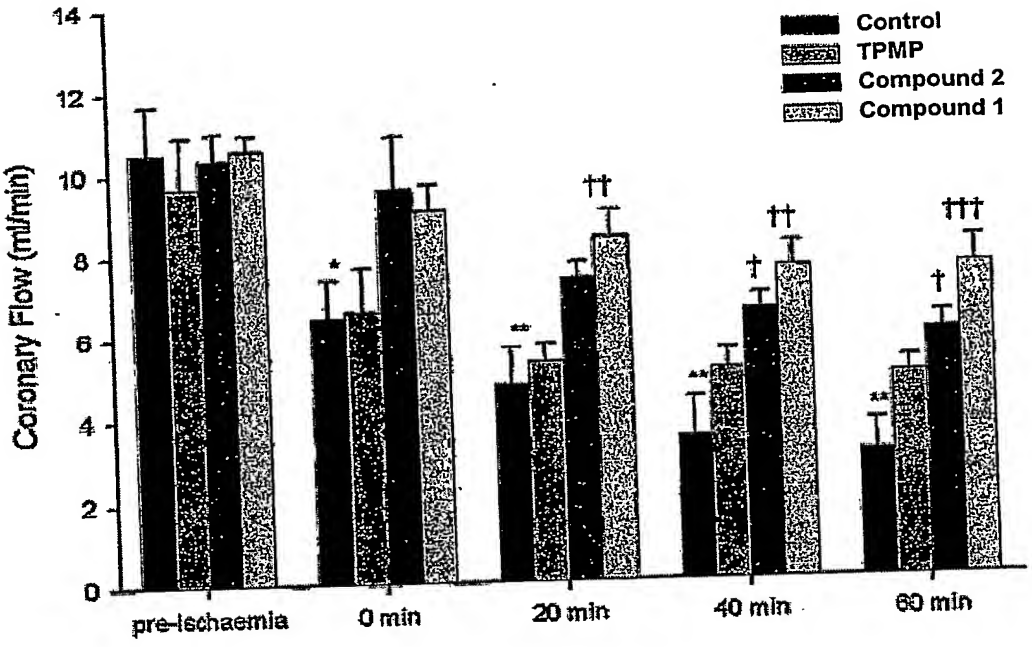

Control
TPMP
Compound 2
Compound 1
Coronary Flow (ml/min)
14
12
10
8
6
4
2
0
pre-ischaemia
0 min
20 min
40 min
60 min

Figure 19

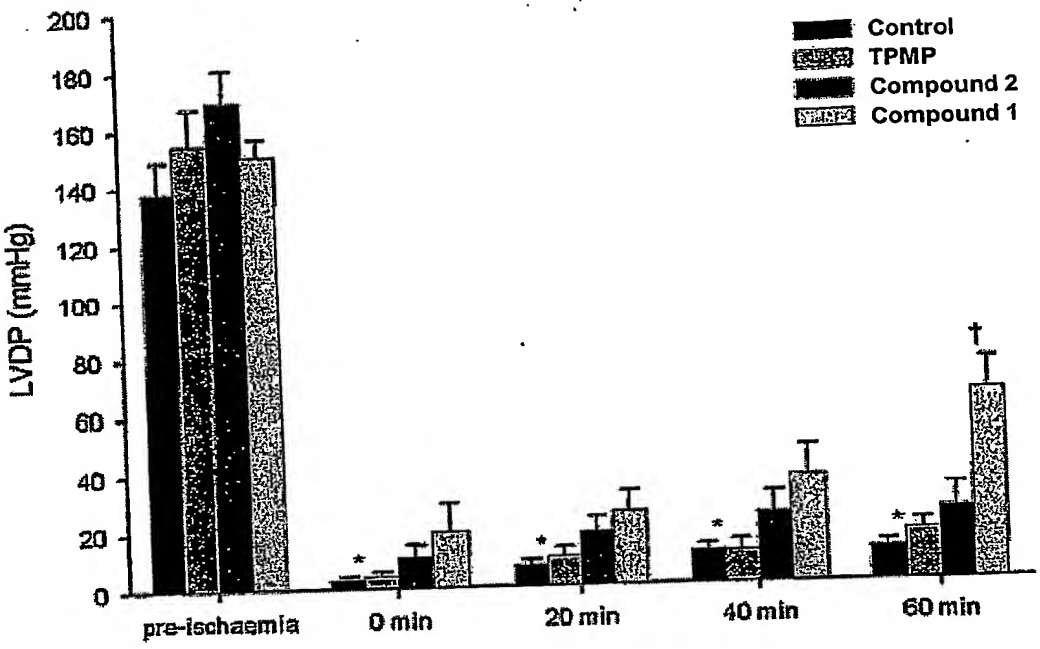

Control
TPMP
Compound 2
Compound 1
LVDP (mmHg)
200
180
160
140
120
100
80
60
40
20
0
pre-ischaemia
0 min
20 min
40 min
60 min
*
*
*
*
†

Figure 20

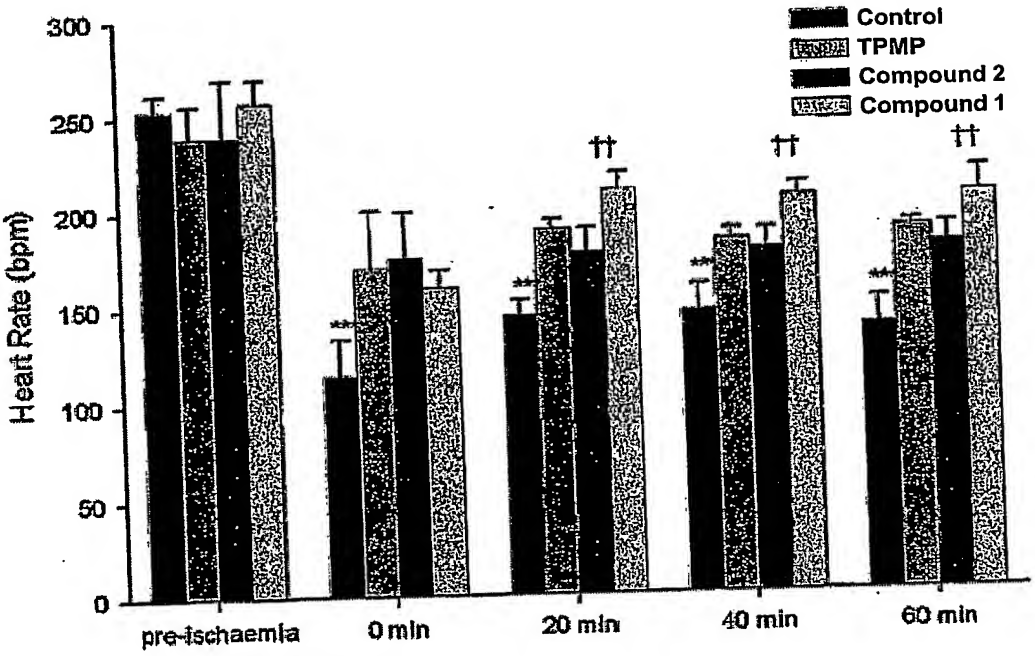

Control
TPMP
Compound 2
Compound 1
Heart Rate (bpm)
300
250
200
150
100
50
0
pre-ischaemia
0 min
20 min
40 min
60 min
**
††

Figure 21

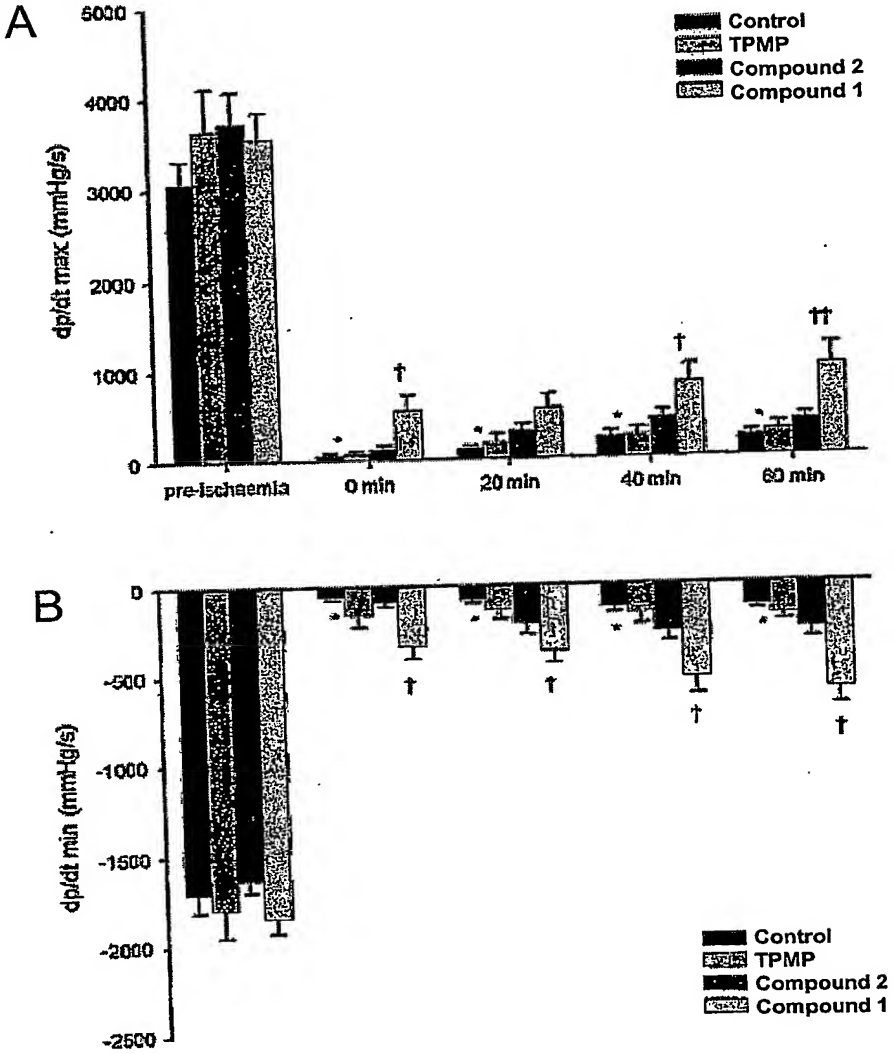

A
5000
4000
3000
2000
1000
0
dp/dt max (mmHg/s)
Control
TPMP
Compound 2
Compound 1
pre-ischaemia
0 min
20 min
40 min
60 min
B
0
-500
-1000
-1500
-2000
-2500
dp/dt min (mmHg/s)
Control
TPMP
Compound 2
Compound 1

Figure 22

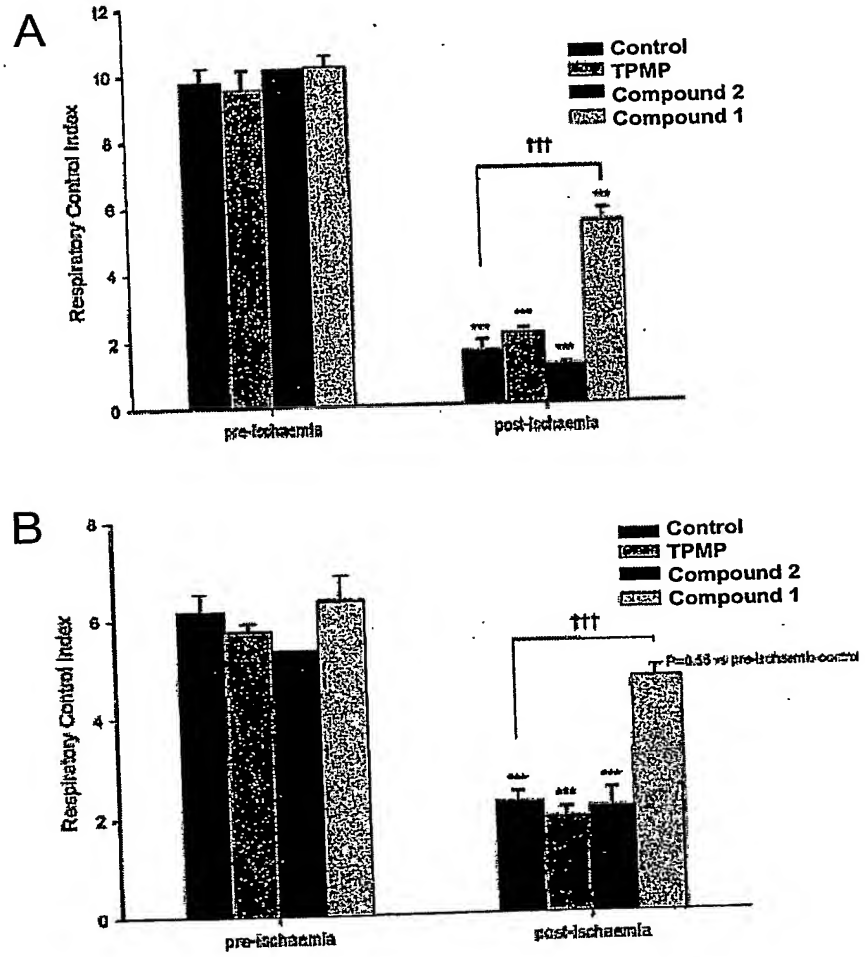

A
Respiratory Control Index
Control
TPMP
Compound 2
Compound 1
†††
***
pre-ischaemia
post-ischaemia
B
Respiratory Control Index
Control
TPMP
Compound 2
Compound 1
†††
pre-ischaemia
post-ischaemia

Figure 23

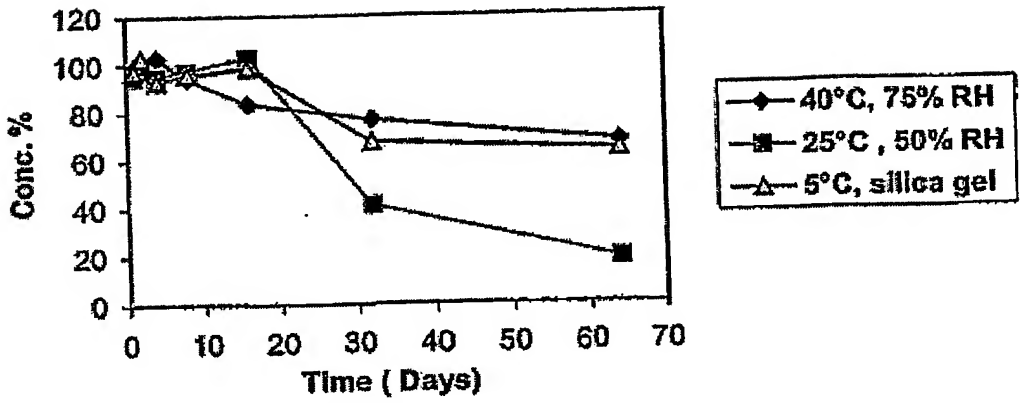

120
100
80
60
40
20
0
Conc. %
0
10
20
30
40
50
60
70
Time (Days)
40°C, 75% RH
25°C , 50% RH
5°C, silica gel

Figure 24

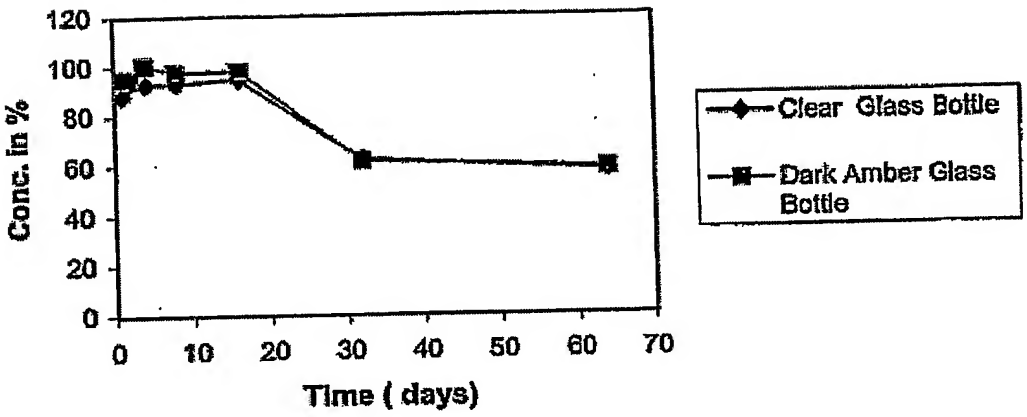

120
100
80
60
40
20
0
Conc. in %
0
10
20
30
40
50
60
70
Time (days)
Clear Glass Bottle
Dark Amber Glass Bottle

Figure 25

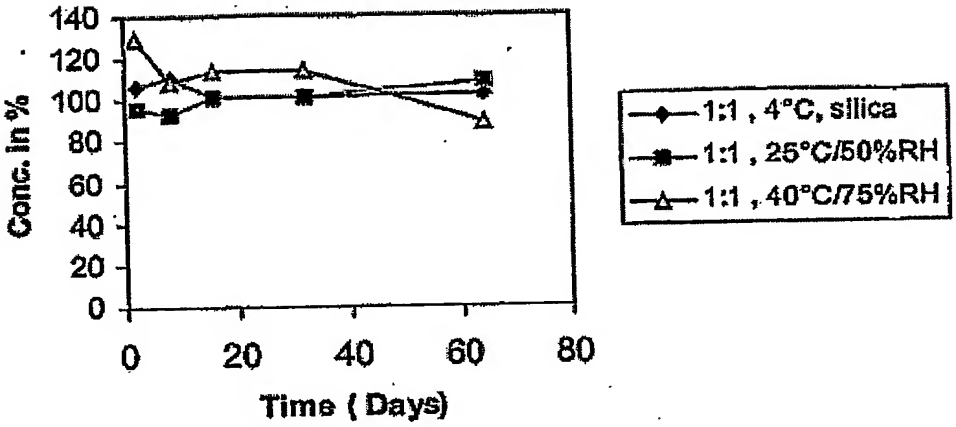

140
120
100
80
60
40
20
0
Conc. in %
0
20
40
60
80
Time (Days)
1:1 , 4°C, silica
1:1 , 25°C/50%RH
1:1 , 40°C/75%RH

Figure26

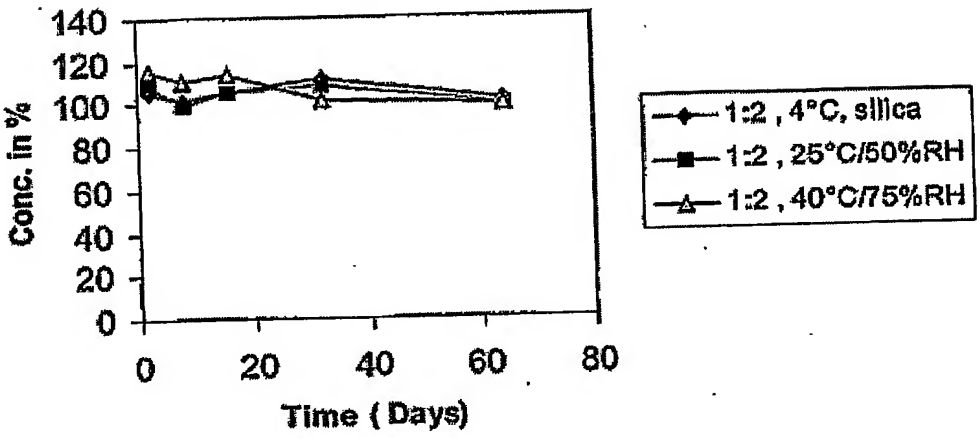
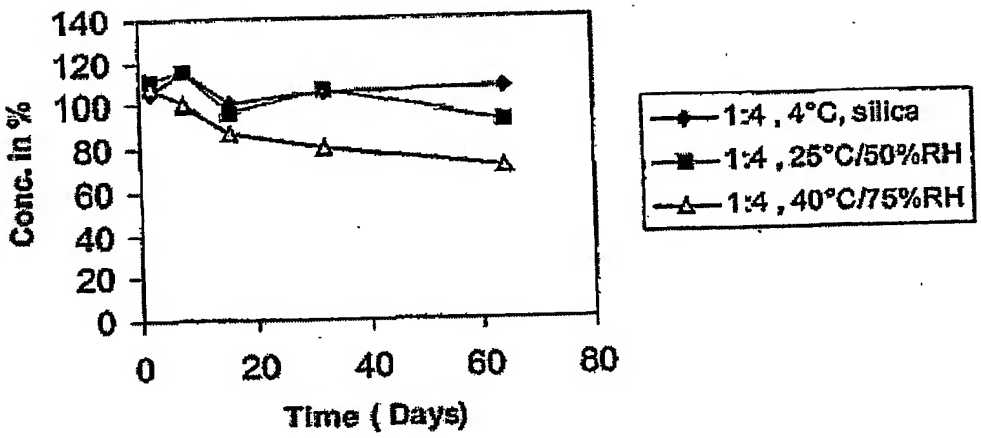

140
120
100
80
60
40
20
0
Conc. in %
0
20
40
60
80
Time (Days)
1:2 , 4°C, silica
1:2 , 25°C/50%RH
1:2 , 40°C/75%RH

Figure 27


140
120
100
80
60
40
20
0
Conc. in %
0
20
40
60
80
Time (Days)
1:4 , 4°C, silica
1:4 , 25°C/50%RH
1:4 , 40°C/75%RH